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# Biological effects of nickel on tropical marine biota to underpin the development of water quality guidelines for metals

Francesca Gissi
*University of Wollongong*

# Biological effects of nickel on tropical marine biota to underpin the development of water quality guidelines for metals

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By

Francesca Gissi

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# Certification

I, Francesca Gissi, declare that this thesis submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Chemistry, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Francesca Gissi

17th June 2019.

# Abstract

Southeast Asia and Melanesia contain the world's largest deposits of nickel lateritic ores and if the mining of these ores are not adequately managed, adverse environmental impacts will occur. Currently, risk assessment tools and water quality guidelines for the tropics are limited due to the sparse research on how contaminants impact tropical biota. Biological effects data for tropical ecosystems were required to assess the risks of contaminant exposure and to derive appropriate water quality guidelines to protect our aquatic ecosystems and to ensure responsible nickel production in Southeast Asia and Melanesia. The aim of this research was to critically review and develop new data on the biological effects of nickel on tropical marine, estuarine, pelagic and benthic species, with a particular focus on corals and their microbiomes. The toxicity of copper, a common contaminant in tropical marine environments, was also investigated to permit comparisons with previous studies.

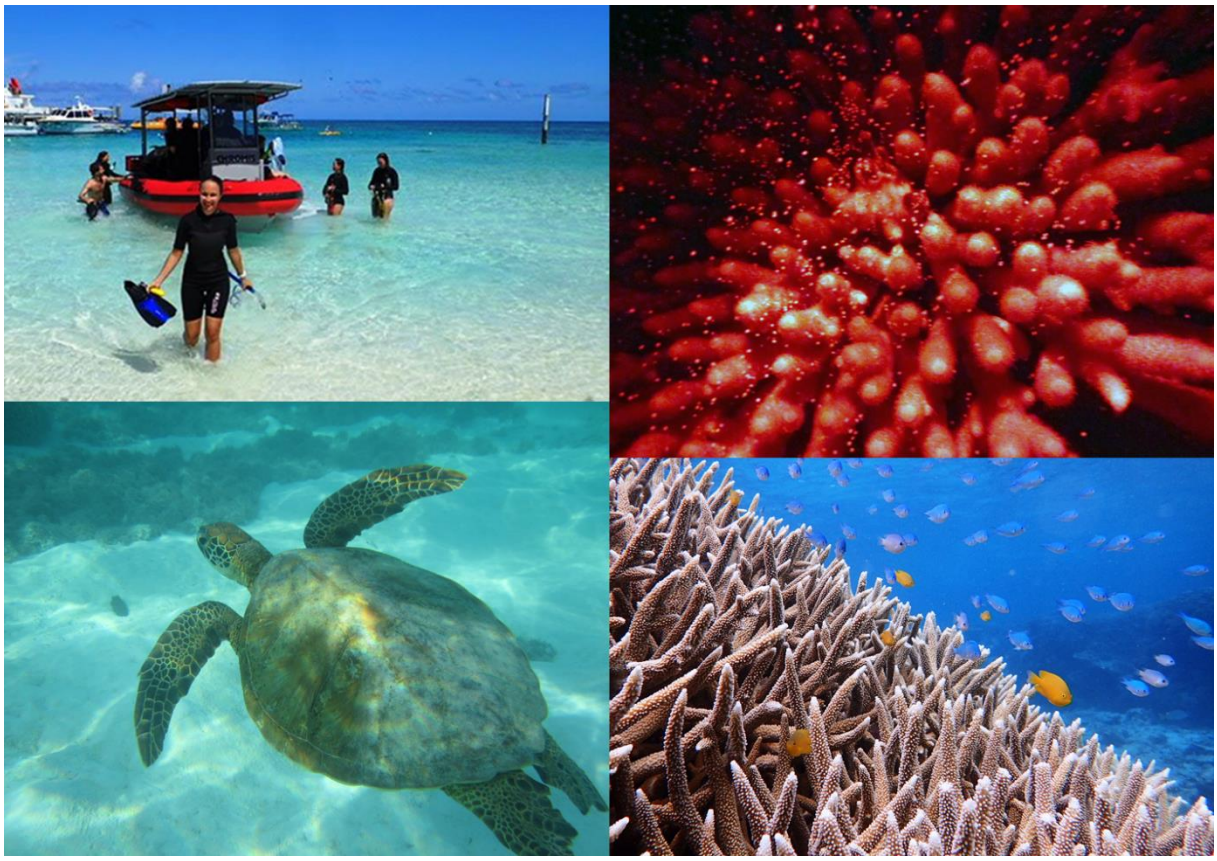
Prior to this research, only six reliable chronic toxicity data (toxicity estimates ranged from 24 – 3700 µg Ni/L) had been reported for nickel to tropical marine species and this was insufficient to derive a water quality guideline. To address this data gap, biological effects data for key tropical marine species including two species of microalgae, a coral algal endosymbiont (*Symbiodinium*), a gastropod, two crustaceans and four species of corals were derived. Toxicity tests utilised chronic endpoints including 72-h growth inhibition for microalgae, 80-96h growth or development for juvenile stages of the gastropod and crustaceans, and 5-h fertilisation success for corals. Toxicity tests with adult corals assessed acute survival over 96 h. For nickel, the 10% effect concentration (EC10) values ranged from 5.5 – 2870 µg Ni/L and the diatom, *Ceratoneis closterium* was the least sensitive species tested. The most sensitive test was the larval development of the copepod *Acartia sinjiensis* and this is the most sensitive tropical marine species to nickel reported so far. Overall, the species tested in this study were more sensitive to copper than nickel, EC10 values ranging from 0.97 – 13 µg Cu/L. The most sensitive species and endpoint was growth of *C. closterium* and the least sensitive was fertilisation in the coral *Platygyra daedalea*.

Corals are fundamental species in tropical marine ecosystems, providing complex structural habitats that support the rich biodiversity found in the tropics. Corals are not yet included in water

quality guidelines and this presents a significant data gap because corals may not be protected by current environmental management plans. This research investigated the effects of dissolved metals (nickel and copper) on early life stages of corals (fertilisation) and determined the effect of dissolved nickel and copper and particulate nickel (bound to sediments) on adult corals and their microbiome. The coral microbiome is fundamental in maintaining the overall health and functioning of corals, therefore it is important to consider how the coral host and its associated microbiota respond when exposed to stressors. Results demonstrated that metal exposure and the combined effect of metals with elevated total suspended sediments has the potential to cause bleaching in corals in addition to altering the coral microbiomes which are inherently linked to coral health. Research into the effects of contaminants on the coral microbiome provides a new understanding of how the microbiome responds when exposed to anthropogenic stressors including increased suspended sediments and metals.

This research has provided high quality data which will contribute to the development of ecologically-relevant water quality guidelines for nickel in tropical marine waters. With the addition of the toxicity data presented in this thesis, there are now sufficient tropical data to derive an interim marine guideline value for nickel that is specific for tropical waters. Data from the literature and generated in this thesis were compiled in a species sensitivity distribution (SSD), providing a total of 11 species representing eight taxonomic groups. The most sensitive species was the copepod, and the least sensitive species was a cyanobacteria. The Protective Concentration value, derived from this SSD, to protect 95% of species (PC95) for slightly-to-moderately disturbed systems was 7.4 μg Ni/L. This value is within the range of values derived for nickel in temperate marine waters by DeForest and Schlekat (2012) (21 μg Ni/L) , the USEPA (8.2 μg Ni/L) and ANZECC/ARMCANZ (7 μg Ni/L). The reliability of PC values derived from SSD methods is based on sample size (number of species for which toxicity data are available), the type of data (chronic, acute etc), and by visual assessment of the SSD to the toxicity data (i.e. good or poor). According to these criteria, the PC95 value generated in this thesis is of moderate reliability. It should be noted that this is not a regulatory value, but a reflection of the continued and ongoing research to produce high quality nickel toxicity data for tropical marine waters. This is valuable because water

quality guidelines are required to manage and reduce environmental risks associated with mining activities in Southeast Asia and Melanesia. In addition, the coral microbiome study provides a foundation for developing such a new tool to monitor environmental impacts.



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Finally, I would like to dedicate this thesis to my late father, Attilio Gissi. From a young age, my father would refer to me as Doctoressa Gissi; he always believed I would be successful and always showed how proud he was of me, which gave me the confidence in my abilities. I hope that I continue to make you proud.

As I worked on the revisions to my thesis, I could feel movements from my baby girl growing inside my tummy. As I reflect on this incredible journey I have had whilst doing my PhD, I think of her and the extraordinary life she will have ahead of her. I am grateful for all the opportunities that I have had and all the wonderful people in my life. I wish that Jackson and I can provide her with all she needs to be the best person she can be and to make all her dreams come true, just like mine have.

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# List of publications

The contents of Chapters 2, 4, 5 and 6 in this thesis have been published. To avoid repetition, the chapters are not exact replicas of the publications. Where the research was done in collaboration with colleagues, this has been acknowledged at the beginning of each chapter as a footnote, however, I wrote each chapter/publication with input from my colleagues. The publications are:

**Gissi, F**., Stauber, J.L., Binet, M.T., Golding, L.A., Adams, M.S., Schlekat, C., Garman, E., Jolley, D.F. 2016. A review of nickel toxicity to marine and estuarine tropical biota with particular reference to the South East Asian and Melanesian region. Environmental Pollution 218:1308-1323. http://dx.doi.org/10.1016/j.envpol.2016.08.089

**Gissi, F**., Stauber, J.L., Binet, M.T., Trenfield, M.A., Van Dam, J.W., Jolley, D.F. 2018. Assessing the chronic toxicity of nickel to a tropical marine gastropod and two crustaceans. Ecotoxicology and Environmental Safety, 159, 284-292. https://doi.org/10.1016/j.ecoenv.2018.05.010

**Gissi, F**., Stauber, J., Reichelt-brushett, A., Harrison, P.L., Jolley, D.F. 2017. Inhibition in fertilisation of coral gametes following exposure to nickel and copper. Ecotoxicology and Environmental Safety 145, 32–41. doi:10.1016/j.ecoenv.2017.07.009.

**Gissi, F**., Reichelt-brushett, A., Chariton, A., Stauber, J.L., Greenfield, P., Humphrey, C., Salmon, M., Stephenson, S., Cresswell, T., Jolley, D.F. 2019. The effect of dissolved nickel and copper on the adult coral *Acropora muricata* and its microbiome. Environmental Pollution, 250:792-806. https://doi.org/10.1016/j.envpol.2019.04.030

As part of a larger program of work, to which research from my thesis has contributed, there was also a review on nickel toxicity to freshwater biota. I contributed to this work and was a co-author on the following publication.

Binet, M.T., Adams, M.S., **Gissi, F**., Golding, L. A., Shlekat, C., Garman, E., Merrington, G., Stauber, J.L. (2017). Toxicity of nickel to tropical freshwater and sediment biota – A critical literature review and gap analysis. Environmental Toxicology and Chemistry, 37, 293-317. DOI: 10.1002/etc.3988.

I also assisted in the development and publication of the chronic copepod bioassay (used in Chapter 4) presented in the following publication.

Binet, M.T., **Gissi, F**., Stone, S., Trin-Quy, C., McKnight, K. 2019. Can scanning and image recognition technology be used to propel tropical copepod larval development tests into the 21st century? Ecotoxicology and Environmental Safety, 180:1-11. https://doi.org/10.1016/j.ecoenv.2019.03.049

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**Gissi, F**.; Gillmore, M.; Stauber, J.; Recihelt-Brushett, A.; Golding, L..; Chariton, A.; Greenfield, P.; Humphrey, C.; Severati, A; Jolley, D. The effect of nickel-contaminated sediments on the coral microbiome. In SETAC North America; 4-8 November 2018, Sacramento, US.

**Gissi, F**.; Gillmore, M.; Recihelt-Brushett, A.; Chariton, A.; Stauber, J.; Golding, L..; Greenfield, P.; Humphrey, C.; Severati, C; Jolley, D. Multiple effects of nickel-contaminated suspended sediment exposure on the coral *Acropora muricata*. In SETAC Asia Pacific; 16-19 September 2018, Daegu, South Korea.

**Gissi, F**.; Recihelt-Brushett, A.; Chariton, A.; Stauber, J.; Stephenson, S.; Greenfield, P.; Jolley, D. Alterations in the coral microbiome following exposure to metals. In Australian Marine Science Association, Canyons to Coasts; 1-5 July; Adelaide, Australia.

**Gissi, F**.; Recihelt-Brushett, A.; Chariton, A.; Stauber, J.; Stephenson, S.; Cresswell, T.; Greenfield, P.; Humphrey, C.; Severati, A.; Jolley, D. The response of corals and the coral microbiome to metal exposure. In SETAC North America; 12-16 November 2017; Minneapolis, US.

**Gissi, F**.; Recihelt-Brushett, A.; Chariton, A.; Stauber, J.; Stephenson, S.; Cresswell, T.; Jolley, D. Impacts of nickel on different lifestages of coral. In: SETAC-AU; 3-6 September 2017; Gold Coast Australia.

**Gissi, F**.; Recihelt-Brushett, A.; Chariton, A.; Stauber, J.; Salmon, M.; Severati, A.; Jolley, D. Using the SeaSim facility for ecotoxicology –testing the effects of Ni and Cu on the adult hard coral *Acropora muricata.* In Australian Marine Science Association (AMSA) conference; 2-6 July 2017; Darwin, Australia.

**Gissi, F**.; Stauber, J.; Jolley, D.; Recihelt-Brushett, A; Trenfield, M.; van Dam, J.; Binet, M.; Schlekat, C.; Garman, E. Filling the Gaps – Nickel toxicity to tropical marine biota. In: SETAC-AU; 4-7 October 2016; Hobart, Australia.

**Gissi, F**.; Reichelt-Brushett, A.; Chariton, A.; Stauber, J.; Jolley, D.; Salmon, M.; Severati, A. Ecotoxicology at SeaSim – Exploring Ni and Cu effects on the adult hard coral *Acropora muricata*. In: SETAC-AU; 4-7 October 2016; Hobart, Australia. Poster.

**Gissi, F**.; Stauber, J.; Jolley, D.; Binet, M.; Trenfield, M.; van Dam, J.; Schlekat, C.; Garman, E. Nickel toxicity to tropical marine biota. In SETAC-EU; 22-26 May 2016. Nantes, France. p 73.

**Gissi, F**.; Stauber, J.; Jolley, D.; Binet, M.; Adams, M.; Golding, L.; Schlekat, C.; Garman, E. Nickel toxicity to tropical marine organisms: Where are the gaps? In: SETAC-AU; 25-28 August 2015. Nelson, New Zealand. p 79.

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# Abbreviations and Nomenclature

| | |
|---|---|
| AIC | Akaike information criterion |
| AIMS | Australian Institute of Marine Science |
| AINSE | Australian Institute of Nuclear Science and Engineering |
| ANOVA | Analysis of variance |
| ANSTO | Australian Nuclear Science and Technology Organisation |
| ANZECC/ARMCANZ | Australian and New Zealand Environment and Conservation Council and Agriculture and Resource Management Council of Australia and New Zealand |
| AR | Analytical reagent |
| ASV | Anodic stripping voltammetry |
| BLM | Biotic ligand model |
| bp | Base pair |
| CL | Confidence limits |
| CSIRO | Commonwealth Scientific and Industrial Research Organisation |
| DOC | Dissolved organic carbon |
| DO | Dissolved oxygen |
| DNA | Deoxyribonucleic acid |
| EC/IC/LCx | Effect, Inhibitory or Lethal Concentration |
| EDTA | Ethylenediaminetetraacetic acid |
| EMP | Earth Microbiome Project |
| FNU | Formazin Nephelometric Unit |
| GBRMPA | Great Barrier Reef Marine Park Authority |
| HC | Hazardous concentration |
| HDPE | High density polyethylene |
| ICP-AES | Inductively coupled plasma – atomic emission spectrometry |
| ICP-MS | Inductively coupled plasma – mass spectrometry |
| INSG | International Nickel Study Group |
| ISO | International Organisation for Standardization |
| IUCN | International Union for the Conservation of Nature |
| JCU | James Cook University |
| LA-ICP-MS | Laser ablation-inductively coupled plasma – mass spectrometry |

| | |
|---|---|
| LDR | Larval development ratio |
| LLDPE | Linear low density polyethylene |
| LOD | Limit of detection |
| LOEC | Lowest observable effect concentration |
| MM | Amplitaq Gold 360 Master Mix |
| µPIXE | Micro Particle-Induced X-ray Emission |
| NEC | No effect concentration |
| NiPERA Inc. | Nickel Producers Environmental Research Association |
| NIST | National Institute of Standards and Technology |
| nMDS | Non-metric multidimensional scaling |
| NOEC | No observable effect concentration |
| OECD | Organisation for Economic Co-operation and Development |
| OTU | Operational taxonomic unit |
| PC | Protective concentration |
| PCR | Polymerase chain reaction |
| PERMANOVA | Permutational multivariate analysis of variance |
| PLC | Programmable logic controller |
| PVC | Polyvinyl chloride |
| RNA | Ribonucleic acid |
| SD | Standard deviation |
| SEAM | South East Asia and Melanesia |
| SeaSim | National Sea Simulator |
| SIMPER | Similarity percentages |
| SRM | Standard reference material |
| SSD | Species sensitivity distribution |
| TOC | Total organic carbon |
| TSS | Total suspended solids |
| TWA | Time weighted average |
| USGS | United States Geological Survey |
| USEPA | United States Environmental Protection Agency |
| WQG | Water quality guideline |

# 1. INTRODUCTION

## Context statement

The primary aim of this study was to provide high quality, chronic nickel toxicity data which will contribute to the development of an ecologically-relevant water quality guideline for nickel in tropical marine waters. This is in response to an increase in mining pressures in the tropics, particularly the mining and production of nickel. Tropical marine ecotoxicity information for nickel, a widespread contaminant in urban and mining areas, is lacking. Data on the effects of nickel on tropical marine species is required to assess hazard, risk and to make informed management decisions.

This chapter critically reviews the value and uniqueness of tropical systems, anthropogenic impacts on aquatic systems, the mining and release of nickel into aquatic environments and the use of water quality guidelines to manage anthropogenic impacts.

## 1.1. Tropical marine ecosystems

The tropics, as defined by the KÖppen classification system, is the region surrounding the Earth's equator, between the Tropic of Cancer and the Tropic of Capricorn. Greater than 75% of the global ecological diversity is found in tropical regions. Tropical seas occupy 36% of the global ocean and hold high cultural and economic value, providing tourism opportunities and food for small island nations (Crame, 2000).

While our understanding of global marine biodiversity is more limited than terrestrial or freshwater studies (Tittensor et al., 2010), a handful of studies have shown that for some key taxa, species richness is highest in the tropics and attenuates towards the poles (Hoeksema, 2007). This gradient is related to the high seawater temperatures and maximum solar irradiation in proximity to the equator which promotes the fast growth of plants, for example seagrasses, mangroves and corals with their associated *Symbiodinium* (Hoeksema, 2007). Mangroves, seagrasses and coral reefs have the highest species richness in the tropics. In addition, they harbour a diverse range of habitats which supports the biodiversity of other marine life from primary producers, zooplankton, larger crustaceans, molluscs, echinoderms and fish (Nagelkerken, 2009).

Tropical systems differ from temperate systems due to their warmer temperatures, high irradiance, high rainfall and high rainfall intensities, strong rainfall seasonality and more frequent pulse events such as cyclones/typhoons and intense storm cells. Periods of high rainfall increase run-off from catchments and potentially cause a higher influx of contaminants, nutrients and sediments into the coastal environment.

### *The region of South East Asia and Melanesia*

The region of the Indo-Pacific is thought to be the epicentre of marine biodiversity (Barber et al., 2006). Many ecosystems of South East Asia and Melanesia (SEAM) are unique, or at least unusual, in their key structural components, the patterns of interconnectedness of these habitats and the high biodiversity that they support. Maintaining connectivity of tropical rivers, estuarine and marine systems is particularly important for many migratory species. The SEAM region including New Caledonia, the Philippines and Indonesia, was identified by Roberts et al. (2002) as the centre

of multitaxon endemism. SEAM as described by the United Nations (UN, 2013) is outlined in Figure 1.1. The region of SEAM is made up of small island nations, mostly developing countries, with relatively poor regulatory frameworks and limited environmental monitoring data. These nations rely heavily on their tropical coasts as a major supplier of their dietary protein and as the main driver of their economy through tourism and fishing (Reichelt-Brushett, 2012). An increase in anthropogenic activities in SEAM could impact the valuable marine ecosystems and the communities dependent on them.

## 1.2. Anthropogenic impacts in tropical marine systems

Globally there has been an increase in anthropogenic activities centred on tropical coastlines which has concerned the scientific community for decades (Gissi et al., 2013; Peters et al., 1997). Terrestrial activities such as mining, agriculture, deforestation and increased urban and industrial development are introducing large quantities of sediment, nutrients and other contaminants into tropical coastal waters (Roberts et al., 2002). Aquaculture for example prawn farming, is particularly common in coastal environments in the SEAM region, with many communities reliant on fish and prawn production systems in modified mangrove or saltmarsh habitats, which often leads to extensive reconfiguration of the coastal landscape. This alters coastal water geochemistry with respect to metal discharges and bioavailability. Increase shipping activity, dredging and oil spills are also a concern (Reichelt-Brushett, 2012). In many tropical developing regions, mining is an important contributor to the economy (Reichelt-Brushett, 2012). The mining, processing and refining of metals have the potential to cause environmental impacts through the release of metals, anions (sulfate and chloride) and sediments. Some mines use in-river disposal of waste rock and tailings, or deep-sea tailings placement, which need to be properly managed to reduce potential environmental impacts (Reichelt-Brushett, 2012). The sources and pathways of contaminants in tropical systems are depicted in Figure 1.2.

It has been hypothesised that toxicity of contaminants may increase in warmer environments due to an increase in rates of metabolism in biota (Chapman et al., 2006), however, detoxification mechanisms may also be upregulated at higher temperatures. Studies have shown that

interactions between temperature and contaminants are both species- and contaminant-specific (Chapman et al., 2006; Kwok et al., 2007; Wang et al., 2014).

## 1.3. Nickel

### 1.3.1. Sources and uses of nickel

Nickel is the 5$^{th}$ most common element on earth and occurs extensively in the earth's crust (Nickel Institute, 2015). In nature, nickel primarily occurs as oxides, sulfides and silicates (Pyle and Couture, 2012). Nickel ores are mined in over 23 countries, and are smelted or refined in 25 countries, including Russia, Canada, New Caledonia, Australia, Indonesia, the Philippines, Cuba, China, South Africa and Brazil. Approximately 1.4 million tonnes of nickel is produced annually and the world demand for nickel is growing at an average rate of 5% per annum (INSG, 2016).

The physical properties of nickel, including corrosion resistance, high strength and durability over a range of temperatures, good thermal and electrical conductivity and alloying ability, have led to its wide use in many industrial activities (Pyle and Couture, 2012). More than 75% of nickel produced is used in the production of alloys (primarily stainless steel) with other metals such as iron, copper and chromium (INSG, 2016). Nickel-containing materials include food preparation equipment, mobile phones, batteries, medical equipment, transport, buildings and power generation (Nickel Institute, 2015).

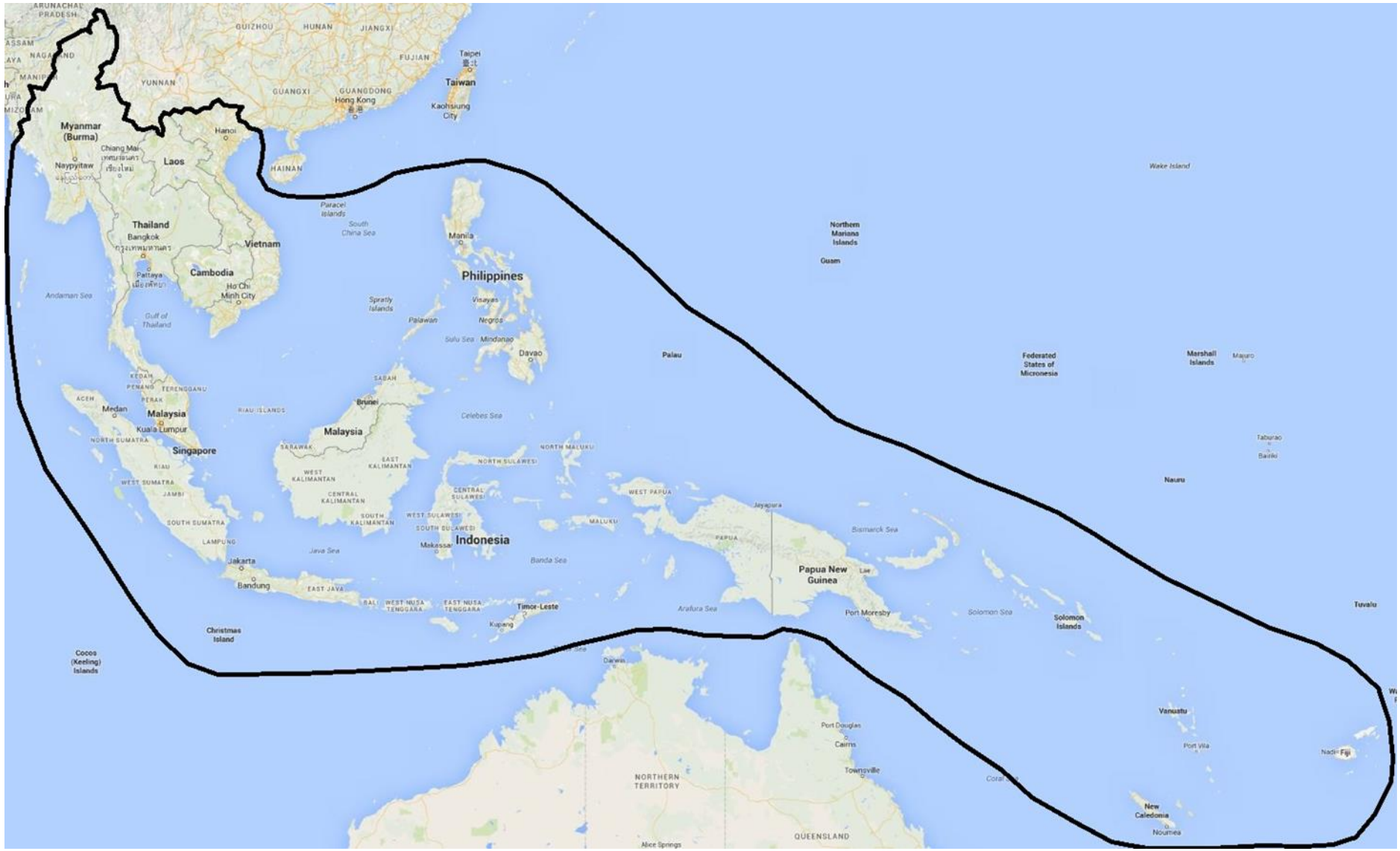



Figure 1.1. Map outlining the region of South East Asia and Melanesia (SEAM)

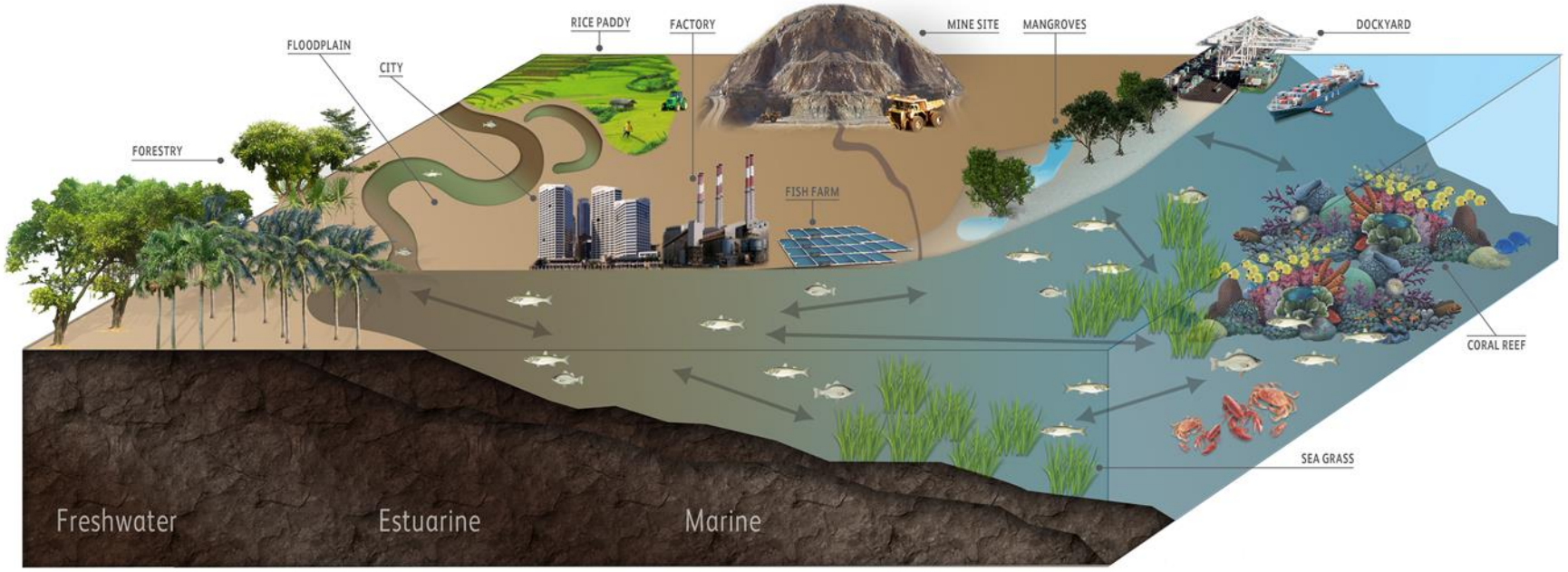



Figure 1.2. Overarching conceptual model depicting the biological and anthropogenic processes that potentially affect tropical ecosystems

### 1.3.2. Nickel mining in tropical regions

Nickel is mined from two main ore types: magmatic sulfide ores or nickel laterites (Mudd, 2010). Magmatic sulfide ores are commonly found in Russia and Canada and have been the primary source of mined nickel for many years. Lateritic ores are more mineralogically complex and lower in nickel grade than sulfide ores, however lateritic ores make up 70% of the world's nickel resources and produce ~ 40% of the global supply (Van der Ent et al., 2013). With the increase in global demand, industry is becoming more reliant on lateritic ore bodies to meet the demand (Bobicki et al., 2014).

The unique geology and climate of tropical countries in SEAM has allowed the formation of nickel laterictic ores. Nickel laterites have a fine dispersive nature and are formed by the extensive chemical and physical weathering of ultramafic rocks under tropical, humid conditions (Mudd, 2010). Ultramafic rocks are derived from ferro-magnesian-rich mantle and are composed of mafic minerals including magnesium, iron and nickel. Ultramafic lateritic soils, typically of tropical countries in Asia-Pacific including Indonesia, the Philippines and New Caledonia, have several extreme chemical properties including unusually high concentrations of iron, chromium, manganese, magnesium and nickel (Van der Ent et al., 2013). The topsoil is typically characterised by low pH, which often results in low pH freshwaters. Metal oxides including iron, manganese and aluminium have been shown to bind nickel in sediments reducing nickel bioavailability (Costello et al., 2016). These characteristics strongly influence the bioavailability and fate of nickel in tropical ecosystems (Van der Ent et al., 2013).

Lateritic ores are typically found in tropical and subtropical regions. In 2015 some of the highest production from nickel lateritic ores (both mining and smelting) occurred on small island nations in SEAM including the Philippines (23%), New Caledonia (8.2%) and Indonesia (7.3%) (USGS, 2016). Over 10 years from 2005 – 2015, the production of nickel from these three countries increased by over 2000% (USGS, 2016, 2006). In 2017,

production in the SEAM region remained high, with Indonesia, New Caledonia and the Philippines producing between 10-19% of the world's nickel (USGS, 2018).

The shallow enrichment of nickel laterites over large surface areas has resulted in large open-cut mining which disturbs large areas of land (Brand et al., 1998; Reichelt-Brushett, 2012). Dissolved and particulate nickel (and other metals such as Co, Cr, Mn) can be introduced into the aquatic environment through surface run-off from the exposed laterites due to weathering, rainfall, tailings spills, accidental spillage during transport (e.g. pipeline rupture), dam seepage and runoff from stockpiles (Figure 1.3 A).

In SEAM, nickel production often occurs adjacent to the coast as this facilitates shipment of nickel-containing substances overseas. From these operations, dissolved and particulate nickel can be released via site run-off, seepage from waste storage facilities, runoff and seepage from slag disposal/storage sites, via solids/slurry from the bag house or as dust from stack and fugitive emissions (Figure 1.3 B).

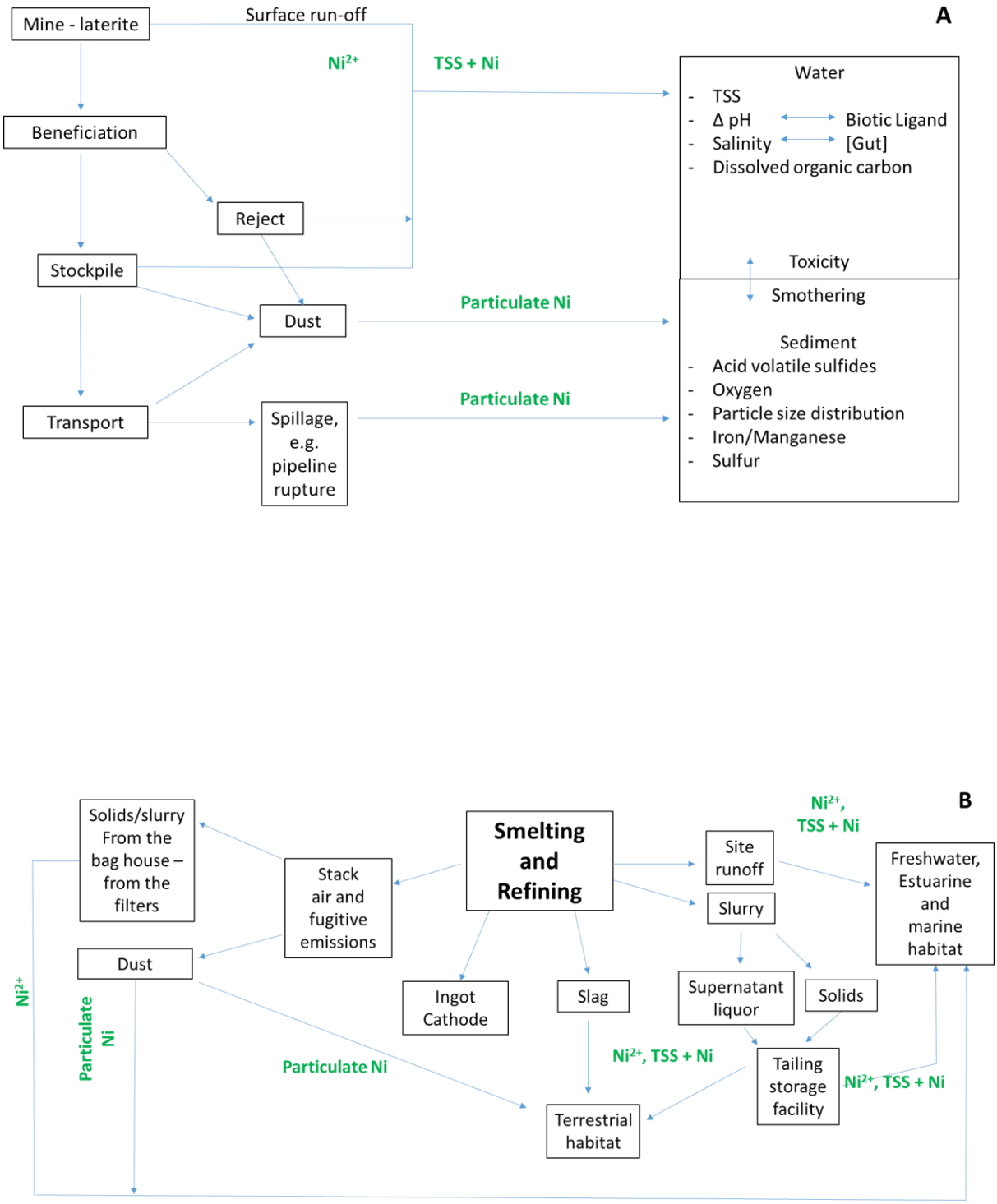



Figure 1.3. Schematic of potential environmental exports of pathways from (A) nickel laterite mining and (B) smelting and refining. TSS = Total suspended solids.

### 1.3.3. Nickel in aquatic systems

Nickel predominately occurs in the oxidation state of +2 and can form stable complexes with inorganic and organic ligands (Eisler, 1998; Pyle and Couture, 2012). In seawater (~pH 8.2), $Ni^{+2}$ is the main form of nickel (~36%), followed by nickel chloro (27%) and carbonate (19%) species (Kumar, 1986). Once nickel has entered an aquatic system, it can be accumulated by biota including phytoplankton and aquatic plants, or deposited in the sediment by precipitation, complexation and adsorption on clay particles, with subsequent uptake in benthic biota (Cempel and Nikel, 2006).

Concentrations of nickel in unimpacted surface marine waters are typically <5 µg Ni/L (Apte et al., 2006; Deforest and Schlekat, 2012). Concentrations of nickel in seawater sampled in the North Pacific ranged from 0.15 – 0.66 µg Ni/L (Bruland, 1980). In some regions, such as New Caledonia, nickel concentrations in soils and aquatic systems are naturally enriched, but mining of lateritic nickel ores can result in the additional input of metals into the surrounding coastal system (Hedouin et al., 2009). In New Caledonia, one study found that an endemic cephalopod had whole-body nickel concentrations up to 6.7 µg/g (Bustamante et al., 2000). Hedouin et al. (2009) found nickel concentrations ranging from 5- 900 mg/kg in marine sediments around New Caledonia. Nickel in the tissues of bivalves living in association with these sediments ranged from 2 – 16 µg/g in oysters and 8 – 52 µg/g in a local clam (Hedouin et al. 2009). Nickel in clams and oysters from other tropical and sub-tropical locations varied from 1.3 – 24 µg/g (Hedouin et al., 2009). While this data is valuable, it is difficult to draw conclusions on toxic effects from tissue/whole body concentrations. Additionally, nickel does not biomagnify in aquatic food chains (Cardwell et al., 2013).

Nickel is known to be an essential nutrient for terrestrial plants and animals and at least eight, nickel-containing enzymes have been identified (Muyssen et al., 2004). In aquatic plants and cyanobacteria, nickel essentiality has been documented in urease and hydrogenase metabolism, however, essentiality in aquatic animals has not yet been confirmed (Muyssen et al., 2004). At higher concentrations, nickel and nickel compounds have been shown to have toxic and carcinogenic effects, believed to be associated with nickel-mediated damage to DNA and proteins

and inhibition of cellular antioxidant defence systems (Eisler, 1998). In freshwater organisms nickel toxicity may occur through disruption of $Ca^{2+}$, $Mg^{2+}$ or $Fe^{2+}$ homeostasis, reactive oxygen species-induced oxidative damage and an allergic-type response of respiratory epithelia (Brix et al., 2017).

It is well established that metal bioavailability is influenced by both water quality parameters (temperature, pH, salinity, conductivity, dissolved organic carbon (DOC), etc.) and the metal-species interaction at the biotic ligand/receptor (Rainbow, 1997). Subsequent toxicity is also determined by the metal's specific mode of action and the organism's behaviour and metabolism, e.g. feeding behaviour, filtration rates and detoxification processes (Chapman, 2008). Measured water quality parameters in tropical marine systems differ significantly from temperate systems due to natural variations in climate and geochemical factors, such as temperature and increased seasonal rainfall, nutrient and DOC concentrations.

There are very few studies available in the literature that investigate the influence of water quality parameters on nickel toxicity to tropical marine biota. Salinity appears to alter nickel bioavailability and toxicity, with increased salinity decreasing nickel toxicity in temperate crabs (Blewett et al., 2015). Only one study showed that pH affects nickel toxicity in a species-dependent manner in two temperate marine organisms, the mysid *Americamysis bahia* and the amphipod *Ampelisca abdita* (Ho et al., 1999). A decrease in pH from 9 to 7 increased nickel toxicity to the mysid, while there was no change in nickel toxicity to the amphipod at different pH values.

There is a paucity of toxicity data on lateritic-based ecosystems of tropical SEAM. Given the unique geochemistry of lateritic soils and the unique climate which affects the bioavailability and fate of nickel (section 1.3.2), this is an important consideration in assessing the hazard and risk associated with nickel mining activities in tropical SEAM.

## 1.4. Risk assessment tools to manage and monitor anthropogenic impacts on aquatic ecosystems

### 1.4.1. Water quality guidelines

Water quality guideline (WQG) values are a management tool that can be used by policy makers, governments, industry and environmental agencies to set acceptable threshold levels to protect aquatic environments from contaminants (Wang et al., 2014). Development

of WQGs requires a multidisciplinary approach involving chemistry, ecotoxicology, bioaccumulation and ecology. Such tools are well developed for temperate regions, however, direct extrapolation of temperate guideline values to the tropics may not be appropriate due to the vast differences in the evolutionarily distinct biota, structural habitats, geochemistry, organic matter and climatic conditions (temperature, rainfall, etc). A study on the differences in pesticide toxicity between temperate and tropical regions distinguished key drivers of differences between organism sensitivities including climatic parameters and ecosystem sensitivity. While pesticide dissipation rates and vulnerability of freshwater ecosystems between temperate and tropical regions did not vary significantly, the differences in the fate and effects for individual pesticides and taxa were significant (Daam and Van den Brink, 2010). Tropical species are often considered to be more sensitive than their temperate counterparts due to differences in genetic composition and metabolism, as well as the unique and high level of biodiversity found in the tropics (Gunnarsson and Castillo, 2018). In the case of metals, it is difficult to accept or reject this theory due to the limited toxicity data for tropical species.

Water quality guideline values are increasingly being derived using species sensitivity distributions (SSDs), which estimate a protective concentration (PC), usually for 95% of species (known as the PC95 value), which corresponds to the 5% hazardous concentration (HC5). The guidance around SSDs and WQG derivation is jurisdiction-dependent. In Europe, 10-15 species from 8-9 taxa are recommended for input into SSDs (EC (European Commission), 2011; OECD, 2011a); in North America, the recommendation is for at least 15 invertebrate and fish species (USEPA, 2005). In Australia and New Zealand, at least eight species representing four taxonomic groups is preferred (Warne et al., 2018). The underlying principle in all jurisdictions is that the greater the number of taxa and species (data) used in an SSD, the more likely the PC95 is to be protective of a broad range of species in the ecosystem. The reliability of WQGs is also dependent on the quality of the data used (Batley et al., 2018; Warne et al., 2018).

### 1.4.2. Aquatic ecotoxicology and water quality guideline development

There is a lack of ecotoxicological studies with tropical marine species and subsequently a lack of available toxicity data for use in WQG development for tropical regions including SEAM (Gissi et al., 2013; Peters et al., 1997; Van Dam et al., 2008). Ecotoxicity tests or bioassays use living organisms as indicators of contaminant bioavailability and subsequent toxicity in aquatic systems (Stauber and Florence, 1987). Toxicity tests are carried out with a range of organisms from different trophic levels, including bacteria, microalgae, invertebrates (e.g. copepods, bivalves and amphipods) and vertebrates (e.g. fish). Organisms exhibit different sensitivities to contaminants as a result of life-histories, feeding behaviours and route of exposure, among other factors (Jin et al., 2015). It is unreasonable to test all organisms from an ecosystem, instead representative species are chosen that reflect the range of sensitivities that may be encountered (Jin et al., 2015).

When using toxicity tests in an ecological risk assessment, it is important to use ecologically-relevant species under environmentally realistic conditions, in order to better predict the impacts of a contaminant or effluent on local biota in the field (ANZECC/ARMCANZ, 2000). Toxicity test protocols are well established for temperate species and the data from these tests have been used to develop environmental guidelines in Australia and New Zealand, Canada, Europe and North America (ANZECC/ARMCANZ, 2000; CCME, 2007; ECHA, 2008; OECD, 2011a; USEPA, 2013).

Acute and chronic toxicity data may be used in WQG development. Definitions of acute and chronic according to the Australian and New Zealand Water Quality Guidelines (as recently revised by Batley et al., 2018) are given below:

*Acute toxicity - a lethal or adverse sub-lethal effect that occurs after a short exposure period relative to the organism's life span*

*Chronic toxicity – an adverse effect that occurs after exposure for a substantial portion of the organism's life span (usually >10%) or an adverse sub-lethal effect on a sensitive early life stage.*

Chronic toxicity data are preferred over acute data for guideline development, because chronic data are a better representation of long-term and population-based effects of contaminants on an organism and therefore provide a higher level of protection (Warne et al., 2018). The preferred order of estimates of chronic toxicity to use in WQG derivation are provided by Warne et al. (2018) and listed in Table 1.1 below. Acute estimates may still be used and can be converted to chronic estimates; acute endpoints are also useful when investigating pulse exposures. Where insufficient chronic data are available (or only EC/IC/LC50 data), Warne et al. (2018) provide guidance for converting data to estimates of chronic NOEC/EC10 data.

Table 1.1. Definitions of toxicity estimates used in water quality guideline derivation; estimates are listed in order of preference

| Abbreviation | Definition |
|---|---|
| **NEC** | No effect concentration |
| **EC/IC/LCx, where x ≤ 10** | Effect, Inhibitory or Lethal Concentration – the concentration to cause 10% effect in the population (e.g. in growth rate, reproduction, survival), relative to controls. |
| **EC/IC/LCx, where x ≤ 20** | Effect, Inhibitory or Lethal Concentration – the concentration to cause 20% effect in the population (e.g. in growth rate, reproduction, survival), relative to controls. |
| **NOEC** | No Observable Effect Concentration – the highest concentration tested which is not statistically different to the controls |

In temperate regions, water quality guideline values (also known as criteria or standards) for nickel have been developed and ranged from 7 – 21 μg Ni/L (ANZECC/ARMCANZ, USEPA, DeForest and Schlekat, 2012). As noted previously, there is insufficient data for tropical marine species to derive similar guideline values.

## 1.5. Conclusion

The SEAM region contains the world's largest deposits of nickel lateritic ores. Environmental impacts may occur if mining operations are not adequately managed. Biological effects data for tropical ecosystems are required to assess the risks of contaminant exposure and to derive WQGs to manage these risks. Currently, risk assessment tools and WQGs for the tropics are limited due to the sparse research on how contaminants impact tropical biota. The following chapter provides a review of available data on the effects of nickel on tropical marine, estuarine, pelagic and benthic species, with a particular focus on SEAM.

# 2. A REVIEW OF NICKEL TOXICITY TO MARINE TROPICAL BIOTA WITH PARTICULAR REFERENCE TO THE SOUTH EAST ASIA AND MELANESIA REGION

## Context statement

As discussed in Chapter 1, the SEAM region is one of the highest producers of nickel in the world, and environmental impacts may occur if mining operations are not adequately managed. One such management tool are WQGs. The development of WQGs for the tropics is hindered due to a lack of relevant data. The aim of this chapter was to compile data on the biological effects of nickel on tropical marine, estuarine, pelagic and benthic species, with a particular focus on SEAM. Data were quality checked and their applicability to WQG development was determined. In addition, toxicity test protocols with relevant species were compiled. Recommendations on testing priorities to fill these data gaps are presented. The work presented in this chapter has been adapted from a published paper[1].

[1] **Gissi, F**., Stauber, J.L., Binet, M.T., Golding, L.A., Adams, M.S., Schlekat, C., Garman, E., Jolley, D.F. (2016). A review of nickel toxicity to marine and estuarine tropical biota with particular reference to the South East Asian and Melanesian region. Environmental Pollution 218:1308-1323. http://dx.doi.org/10.1016/j.envpol.2016.08.089.
As first and corresponding author the vast majority of work presented in this chapter and in the paper is a direct result from my PhD research. I did the literature review, compiled toxicity data and test information, adapted the quality check criteria (from ANZECC/ARMCANZ,2000 and Warne et al., 2018), conducted the data quality check and gap analysis, and prepared the manuscript. The data and manuscript were reviewed by all co-authors.

## 2.1. Introduction

Anthropogenic activities including industry, urbanisation and land development (agriculture and mining) are having a major impact on tropical coasts through an increase in sedimentation and input of nutrients and chemical contaminants (Peters et al., 1997). Biological effects data are required to understand the sensitivity of tropical biota to contaminants, and this together with appropriate exposure data can aid risk assessment of contaminants in tropical systems.

Limited studies using species sensitivity distributions (SSDs) have shown that tropical marine species are no more or less sensitive to contaminants than their temperate counterparts and that it is difficult to predict sensitivity between different climatic regions (Chapman et al., 2006). Wang et al. (2014) found only small differences in the acute toxicity of chemicals between tropical and temperate marine biota. Based on the acute data compiled in that study, the authors found that tropical species were more sensitive to copper, mercury, zinc, phenol and pentachlorophenol than temperate species, while temperate species were more sensitive to nickel, chromium, lead, tributyltin and unionised ammonia than tropical species. These conclusions were based on limited tropical acute data and therefore these generalisations cannot be made for all species, all endpoints, or all chemicals. From their SSDs, hazardous concentrations for nickel to 10% of species (HC10) were determined, giving values of 658 (557-767) µg Ni/L (95% confidence limits, CL) for temperate species, and 1560 (366-3060) µg Ni/L for tropical species (Wang et al., 2014). While this suggests that tropical species may be less sensitive to nickel than temperate species, there is considerable uncertainty (overlap of the 95% confidence limits). Similar comparisons with chronic nickel data have not been published and the toxicity of nickel to key unique tropical taxa such as corals and seagrasses has not been studied.

Ecologically-relevant risk assessment tools are needed to assess risks associated with nickel mining in tropical marine and estuarine ecosystems of SEAM. It may not be appropriate to apply tools developed for temperate regions to the tropics due to the vast

differences in the evolutionarily distinct habitats and biota, which may have different inherent sensitivities to nickel, as well as different geochemical and climatic conditions (temperature, rainfall, seasonality) of tropical regions which can influence the bioavailability of nickel (Chapter 1, section 1.4.1.). There is a lack of understanding of both exposure and effects of nickel on tropical marine species, especially those in key habitats such as mangroves, seagrasses and coral reefs. While it is well known that water quality parameters such as pH, salinity and dissolved organic carbon influence nickel bioavailability in temperate systems, these findings have not been validated in tropical ecosystems (Blewett and Wood, 2015; Ho et al., 1999). It is also difficult to uncouple the effects of temperature from the intrinsic differences in sensitivities between tropical and temperate species (Gunnarsson and Castillo, 2018). To date, no comparative data for chronic nickel toxicity between temperate and tropical marine species have been reported.

The objectives of this chapter were to firstly compile and critique existing nickel toxicity data for tropical marine and estuarine species, specifically tropical species that are relevant to the SEAM region, and to determine their quality and therefore their applicability to water quality guideline development. Existing tropical marine and estuarine aqueous and whole-sediment toxicity tests were also reviewed, with a particular focus on species relevant to the SEAM region. Following this, the additional nickel data and toxicity tests that may be required to help decrease uncertainty when assessing the risks of nickel exposure in SEAM were identified. The effects of water chemistry parameters on nickel toxicity to tropical marine biota were also reviewed. This is important with respect to development of WQGs and also in determining the applicability of bioavailability-based models such as the biotic ligand model (BLM) to risk assessment in the tropics.

## 2.2. Methods

Tropical biota were defined as test species isolated from tropical regions, and/or having a natural geographical distribution between the Tropic of Cancer and the Tropic of Capricorn. Tropical toxicity tests were those that were conducted at temperatures ≥25°C. Throughout

this chapter, toxicity tests refer to laboratory bioassays carried out under controlled conditions. Studies were classified as marine if the test species was predominantly found in marine habitats and the toxicity test was conducted at salinities ≥28‰ (USEPA, 2013).

The relevance of the toxicity test species and nickel data to the SEAM region (described in Chapter 1, Section 1.1) was based on the geographical distribution and origins of the test species using literature searches or through the use of databases including Ocean Biogeographic Information System (OBIS), FishBase, and AlgaeBase.

### 2.2.1. Nickel toxicity data compilation

Compiled nickel toxicity data were screened for reliability, i.e. quality, and then for relevance to SEAM. Databases searched for nickel effects data included Web of Science, Scopus and Google Scholar. Nickel effects data included statistical estimates of toxicity such as EC/IC10 (the concentration that causes a 10% effect or inhibition in the population relative to the control), EC/IC50 (the concentration that causes a 50% effect or inhibition in the population relative to the control), NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration). Additional data were compiled including species and life-stage of test organisms, exposure duration and test type (acute or chronic), endpoint (e.g. survival, growth, fertilisation), the type of nickel salt used and key water quality parameters including test temperature, pH, DOC and salinity.

### 2.2.2. Tropical toxicity test compilation

Toxicity tests for tropical marine species, with a particular focus on species relevant to SEAM, were compiled. This was done to identify tests which may be used to fill the gaps in nickel toxicity data. Information on the test species and test methods were compiled for each toxicity test.

### 2.2.3. Quality assessment of data

Compiled nickel toxicity values (e.g. EC10/50, NOEC, LC50, LOEC values) were assessed using a quality checklist modified from ANZECC/ARMCANZ (2000), Batley et al. (2018) and

Warne et al. (2018). These criteria are similar to those used in Europe in the Criteria for reporting and evaluating ecotoxicity data (CRED) (Moermond et al., 2016). The checklist criteria and the scoring system used are presented in Table 2.1. Data were scored as high quality (QA1) if they answered yes to the two questions:

Question A: Was the measured endpoint ecologically relevant (e.g. lethality, immobilisation, growth, development, population growth or reproduction)?

Question B: Were nickel concentrations measured?

and the data scored ≥80% (based on the remaining criteria identified in Table 2.1). Remaining data were scored as QA2 or QA3, or fail, depending on whether they met the above questions and based on their quality assessment score (for descriptions see Table 2.1). Endpoints or toxicity data were considered to be ecologically-relevant where there was a clear link to a species' ecological competitiveness (e.g. lethality, immobilisation, growth, development, population growth and reproduction) (Warne et al., 2018).

Table 2.1. Data quality assessment and scoring criteria (adapted from Batley et al., 2018 and Warne et al., 2018)

| Question | | Score |
|---|---|---|
| **A** | Was the measured endpoint ecologically-relevant (i.e. acceptable endpoints as defined by Batley et al., 2018) and applicable to guideline development? Note: If this is not met, data automatically fails | Pass/Fail |
| **B** | Were nickel concentrations measured? Note: if this is not met, data may still pass as QA3, if overall score is >80%. | Pass/Fail |
| | **Data Quality Assessment** | |
| **1** | Was the exposure duration stated? | 10, 0 |
| **2** | Was the biological endpoint (e.g. immobilisation or population growth) stated and defined (10 marks)? Award 5 marks if the endpoint is only stated | 10, 5 or 0 |
| **3** | Was the biological effect stated (e.g. LC or NOEC)? | 5 or 0 |
| **4** | Was the biological effect quantified (e.g. 50% effect, 25% effect)? | 5 or 0 |
| **5** | Were appropriate controls (e.g. a no-toxicant control and/or solvent control) used? | 5 or 0 |
| **6** | Was each control and chemical concentration at least duplicated? | 5 or 0 |
| **7** | Were test acceptability criteria stated (e.g. mortality in controls must not exceed a certain percentage) OR inferred (e.g. tests used USEPA, OECD, etc. methods which have validation criteria) (award 2 marks). | 5, 2 or 0 |
| **8** | Were the characteristics of the test organism (e.g. length, mass, age) stated? | 5 or 0 |
| **9** | Was the type of test medium used stated? | 5 or 0 |
| **10** | Was the type of exposure (e.g. static, flow-through) stated? | 4 or 0 |
| **11** | Was the experiment replicated? | 4 or 0 |
| **12** | Were parallel reference toxicant toxicity tests conducted? | 4 or 0 |
| **13** | Was there a concentration-response relationship either observable or stated? | 4 or 0 |
| **14** | Was an accepted statistical method or model used to determine the toxicity? | 4 or 0 |
| **15** | For NOEC/LOEC data was the significance level 0.05 or less?<br>OR<br>For LC/EC data was an estimate of variability provided? | 4 or 0<br><br>4 or 0 |
| **16** | Was pH measured?<br>Award 3 marks if measured during the test and values stated.<br>Award 1 mark if measured but values not stated or if they are measured and values are stated for the dilution water only. | 3, 1 or 0 |
| **17** | For metals tested in freshwater, were the following parameters measured? | |
| | (i) hardness | 3, 1 or 0 |
| | (ii) alkalinity | 3, 1 or 0 |
| | (iii) dissolved organic carbon concentration | 3, 1 or 0 |
| | (iv) conductivity | 3, 1 or 0 |
| | Award 3 marks for each variable measured during the test and values stated. | 3, 1 or 0 |

Table 2.1. Data quality assessment and scoring criteria (adapted from Batley et al., 2018 and Warne et al., 2018)

| | Question | Score |
|---|---|---|
| | Award 1 mark for each parameter measured but values not stated. | |
| **18** | For marine and estuarine water, was the salinity/conductivity measured and stated? | 3 or 0 |
| **19** | For tests not using aquatic macrophytes and alga, was the dissolved oxygen content of the test water measured during the test?<br>Award 3 marks if measured during the test and values stated.<br>Award 1 mark if measured but values not stated. | 3, 1 or 0 |
| **20** | For metals tested in sediments, were the following parameters measured? | |
| | Particulate organic carbon | 3, 1 or 0 |
| | Particle size (e.g. <63 µm or silt/clay) | 3, 1 or 0 |
| | Acid-volatile sulfide (AVS) and 1-M HCl extractable metals | 3, 1 or 0 |
| | Dissolved ammonia and sulfide in pore water or overlying water | 3, 1 or 0 |
| | Salinity and conductivity (overlying water, marine/estuarine sediments) | 3 or 0 |
| | Was the alkalinity, hardness or concentrations of Ca and Mg measured in the overlying water? Were known interacting elements on bioavailability measured (e.g. Cl for Cd?)<br>Award 3 marks for each variable measured during the test and values stated.<br>Award 1 mark for each parameter measured but values not stated or if they are measured and values are stated for the dilution water only. | 3 or 0 |
| **21** | Was the temperature measured and stated (3 marks)? | 3 or 0 |
| **22** | Was the grade or purity of the test contaminant stated (3 marks) | 3 or 0 |
| | Total possible score for various types of data<br>Tests with macrophytes and algae<br>Freshwater = 103 Freshwater = 100<br>Marine/estuarine = 97 Marine/estuarine = 94<br>Sediment = 112 Sediment = 109<br>Quality score = [Total score/Total possible score] x 100 | |
| | Final Scoring<br>**QA1 –** Passes A and B, achieves ≥80% in data quality assessment<br>**QA2 –** Passes A and B, achieves ≥ 50 % and <80% in data quality assessment<br>**QA3 –** Passes A, fails B, achieves ≥80% in data quality assessment<br>**Fail –** Passes A , fails B, achieves <80% in data quality assessment<br>**Fail** – Fails A | |

### 2.2.4. Species sensitivity distributions

To identify sensitive taxa, all tropical data and SEAM-only data were fitted to species sensitivity distributions (SSDs) using BurrliOz Version 2 software (Barry, 2014). SSDs were generated using chronic data only. The preferred order of statistical estimates of chronic toxicity to use in an SSD is: No effect concentration (NEC); effect, inhibition or lethal concentration (EC/IC/LCx where x ≤10); EC/IC/LC 15-20; and NOEC (Warne et al., 2018) (discussed in Chapter 1, Section 1.4.1). If insufficient chronic NEC, EC/IC/LC (≤10 to ≤20) and NOEC data are available, chronic LOEC and EC/IC/LC50 data can be converted to chronic NOEC values by dividing by 2.5 and 5, respectively. There were two instances where this was necessary. For the copepod *Acartia pacifica*, the chronic LOEC was converted to an estimated NOEC by dividing by 2.5. For the coral *Platygyra daedalea,* the chronic EC50 was divided by 5. Where there were multiple endpoints for one species, the data for the longest exposure duration were selected first and then the lowest effects concentration was chosen to be included in the SSD. If there was more than one effect concentration for the same species, same life-stage, endpoint and exposure duration, the geometric mean was used. There was one instance where this occurred, for the microalga *Nitzschia closterium* (Table 2.2, now known as *Ceratoneis closterium*).

The type of distribution that is fitted to the data is automatically determined by BurrliOz, and will depend on the number of species and taxonomic groups (Barry, 2014; Warne et al., 2018). Here, chronic toxicity datasets with eight or more data were fitted to a Burr Type III distribution. Smaller datasets were fitted to other distributions, e.g. log-logistic.

### 2.2.5. Gap analysis

Data gaps were identified based on the existing spread of available data across taxa, with particular focus on ensuring that there were sufficient high quality chronic data which could be used to derive a WQG (ANZECC/ARMCANZ, 2000; USEPA, 2005; Wheeler et al., 2002), the ecological relevance to SEAM, and the sensitivity of particular taxa based on other nickel studies from temperate or tropical marine or freshwater studies (e.g. Niyogi et al., (2014))

found a freshwater snail to be highly sensitive to nickel). A further literature search was undertaken to identify the ecological importance of key taxa to the SEAM region, based on endemism and biodiversity. This was challenging due to the lack of information on biodiversity of marine and estuarine species, particularly for those relevant to SEAM (Tittensor et al., 2010). Appendix A, Table A10 summarises information on the diversity and relevance of certain marine taxa to SEAM. This was used to prioritise future testing to obtain nickel toxicity data for tropical species from this region.

## 2.3. Results and Discussion

### 2.3.1. Tropical marine nickel toxicity data

Nickel toxicity data (acute and chronic) were compiled for tropical marine biota including microalgae, crustaceans, echinoderms, annelids, cnidarians and fish. A total of 51 data were found for 30 different species (Table 2.2 - Table 2.7). Eighteen of these species were confirmed to be relevant to the SEAM region. The most sensitive species (based on chronic endpoints) included crustaceans, molluscs, cnidarians and echinoderms. Of the species not found in SEAM, shrimps and mysids were the most sensitive to nickel, with LC50 (50% lethality) values ranging from 7 - 152 µg Ni/L (Table 2.3). One species of tropical marine polychaete was also found to be relatively sensitive to nickel, with 50% inhibition of larval settlement over 96 h (EC50) of 162 µg Ni/L (Table 2.5).

Of the SEAM-relevant species, the most to least sensitive species were: bivalves> sea urchins> anemones> snails > corals> microalgae. These toxicity tests were all conducted at similar water chemistries, i.e. at pH ≥8 and salinities ≥34‰, with the exception of one copepod discussed below. Another exception was unpublished data provided by Jocelyn Senia (AquaBiotech) including data for scallop and seashell species which were conducted at 24°C. Both species are endemic to New Caledonia and the temperatures used in the tests reflected the winter seawater temperatures during the spawning period (Jocelyn Senia, pers comm). The 96-h development of four species of bivalves was inhibited by 10% (IC10) between 12 and 120 µg Ni/L (Table 2.4). Sea urchins have also been shown to be sensitive

to nickel; three different species were found in the literature with EC50 values (48-h inhibition of development) ranging from ~ 60 to 120 µg Ni/L (Table 2.5). One species of anemone was also found to be sensitive to nickel; the 28-d reproduction of this species was inhibited by 10% (IC10) at 66 µg Ni/L (Table 2.5). Data were found for one species of snail, relevant to SEAM. This study measured the acute 96-h survival of adult and larval snails. Snail larvae were almost 180 times more sensitive to nickel than adults, with EC50 values of 200 µg Ni/L and 35500 µg Ni/L, respectively (Table 2.4). Toxicity data were found for three different species of corals. The effect of nickel on fertilisation success was measured in two coral species and effects observed between 1420 and >2000 µg Ni/L (Table 2.6). The survival and settlement of coral larvae in a third species was measured following exposure to nickel and the survival was inhibited by 50% (EC50) at 9000 µg Ni/L (Table 2.6). For two species of microalgae and one cyanobacterium, the IC10 values (10% inhibition in population growth rate) ranged from 340 to 7000 µg Ni/L (Table 2.2).

One test with a copepod was conducted at pH 7.9 – 8.3 and salinity of 25‰. This copepod species is relevant to SEAM and has a marine and estuarine distribution. In this test, the effects of nickel on egg production over 10-d gave a LOEC value of 100 µg Ni/L (Table 2.3). This suggests that copepods may also be sensitive to nickel, although very little information is available on the effects of salinity on nickel toxicity to tropical, marine organisms, and it is possible that nickel toxicity to this copepod may vary if this test was carried out at 35‰.

*Nickel toxicity data quality assessment*

For QA1 data, only five taxonomic groups were represented, and 50% of the data presented here were for cyanobacteria and microalgae (Figure 2.1 A), with only 12.5% each for crustaceans, echinoderms, annelids and cnidarians. Seven out of the eight data were chronic and all data except the crustacean (a mysid) and polychaete were relevant to SEAM. The inclusion of QA2 data only increased the number of taxonomic groups to six, with the addition of one gastropod (Figure 2.1 B). Again, cyanobacteria and microalgae represented

the majority of the data (36%), and only the crustaceans (mysid and shrimp) and polychaetes were not relevant to SEAM. Of the QA1 and QA2 data, 73% were chronic data.

If all data graded in the quality assessment are included (QA1, QA2 and QA3), a total of seven taxonomic groups are represented. Cyanobacteria and microalgae still represent the majority of the data (22%), along with bivalves (22%), followed by sea urchins (17%). The remaining taxonomic groups, crustaceans (copepods, mysid, shrimp), gastropods, annelids (polychaete) and cnidarians (anemone and coral), each represented 5.5% of all the data. All QA3 data were relevant to SEAM. Of the data presented here, 78% are chronic data (Figure 2.1 C).

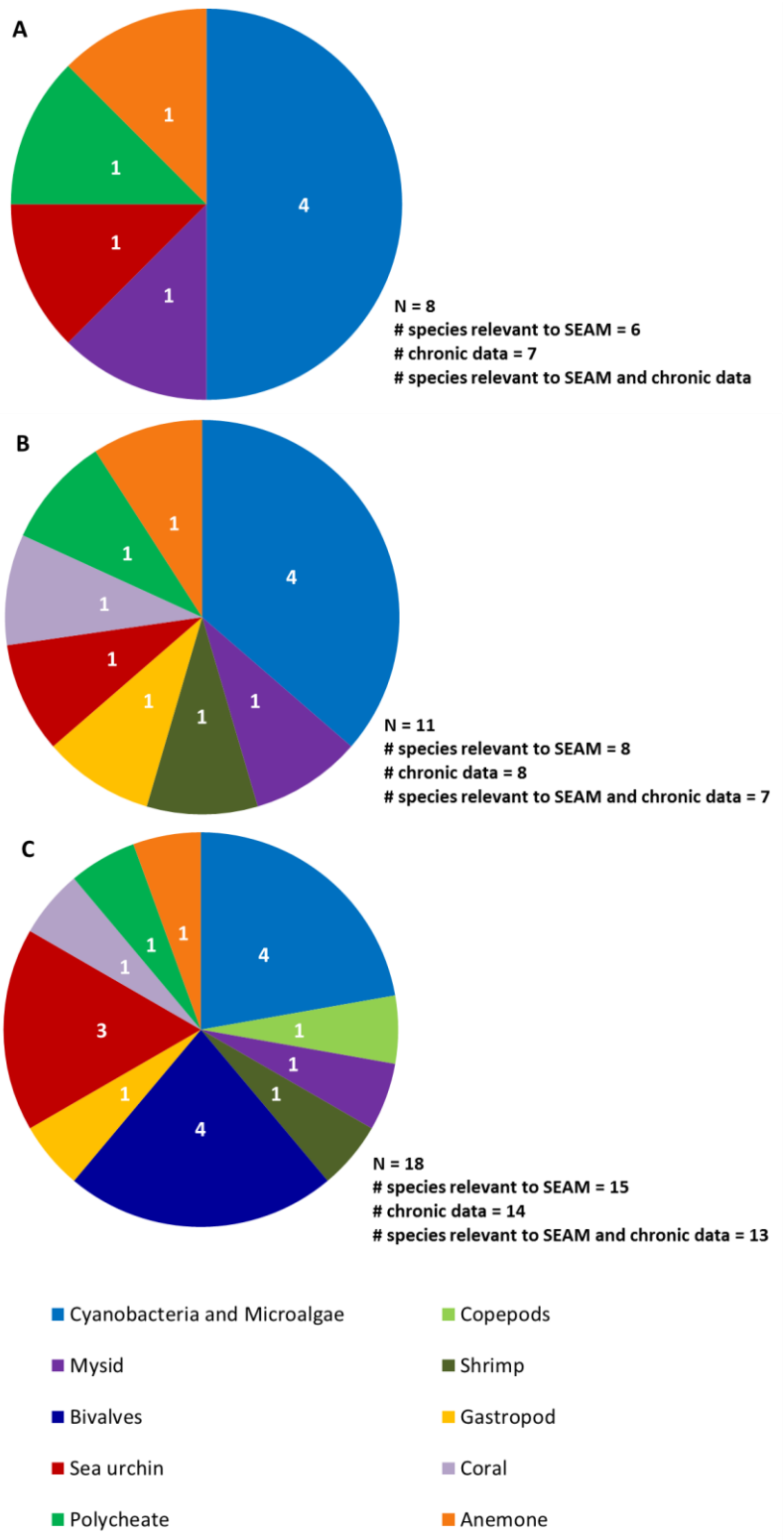



Figure 2.1. Available number of data for use in tropical marine species sensitivity distributions for nickel toxicity data (acute and chronic) scored as QA1 (A), QA1 and QA2 (B), and QA1, QA2, and QA3 (C). Note, there are two data for the same species of microalga, *N. closterium*, the geometric mean of these data was taken for input into species sensitivity distributions.

Table 2.2. Nickel toxicity data for tropical marine microalgae (rounded to 2 significant figures). Grey shading indicates relevance to SEAM; no shading indicates species is not relevant to SEAM[a,b].

| | | | | | Water quality parameters | | | | Toxicity values (µg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Growth phase/initial cell density** | **Endpoint** | **Acute/chronic** | **Test duration** | **Temp (°C)** | **pH** | **Salinity (‰)** | **DOC (mg/L)** | **EC10** | **EC50** | **Data quality** | **Reference** |
| **Cyanobacteria** | | | | | | | | | | | | |
| ***Cyanobacteria Cyanobium* sp.** | 6 x$10^3$ cells/mL | growth rate | chronic | 72 h | 25 | 8 | 33 | NR | 3700 | 2300 | QA1 | Alquezar and Anastasi (2013) |
| **Microalgae** | | | | | | | | | | | | |
| ***Nitzschia closterium* [c]** | log-phase. 3-5 x $10^4$ cells/mL | growth rate | chronic | 72 h | 27 | ~ 8.1 | ~ 35 | NR | NR | >500 | QA2 | Florence et al. (1994) |
| ***Nitzschia closterium* [c e]** | log-phase. 1-3 x $10^3$ cells/mL | growth rate | chronic | 72 h | 27 | 8.1 ± 0.2 | 35 | 1 ± 0.2 | 6100 (3600-9300) | >9500 | QA1 | CSIRO. Gissi et al. Unpublished. |
| ***Nitzschia closterium* [d e]** | log-phase. 1-3 x $10^3$ cells/mL | growth rate | chronic | 72 h | 27 | 8.1 ± 0.2 | 35 | 1 ± 0.2 | 2900 (1500-4200) | 7600 (7060-8200) | QA1 | |
| ***Isochrysis* sp.** | log-phase. 1-3 x $10^3$ cells/mL | growth rate | chronic | 72 h | 27 | 8.1 ± 0.2 | 35 | 1 ± 0.2 | 340 (30-510) | 1700 (1600-1800) | QA1 | |

[a] All toxicity values are measured, dissolved Ni, unless otherwise stated
[b] All tests used $NiCl_2$ or $NiCl_26H_2O$, unless otherwise stated
[c] Now known as *Ceratoneis closterium,* grown in G2 medium
[d] Now known as *Ceratoneis closterium,* grown in F2 medium
[e] The geometric mean was taken for these two data and used in the species sensitivity distributions

Table 2.3. Nickel toxicity data for tropical marine and estuarine crustaceans (rounded up to 2 significant figures). Grey shading indicates relevance to SEAM; no shading indicates species is not relevant to SEAM[a,b].

| | | | | | Water quality parameters | | | | Toxicity values (µg/L) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Life stage** | **Endpoint** | **Acute/ chronic** | **Test duration** | **Temp (ºC)** | **pH** | **Salinity (‰)** | **DOC (mg/L)** | **EC10** | **EC50** | **LOEC** | **Data quality** | **Reference** |
| **Copepods** | | | | | | | | | | | | | |
| ***Acartia pacifica*** | adult females | egg production | chronic | 10 d | 25 | 7.9-8.25 | 25 | NR | | | 100 | QA3 | Mohammed et al. (2010) |
| | eggs | egg hatching success | acute | 48 h | | | | | tox values not calculated: egg hatching success was significantly reduced at 10 µg Ni/L | | | | |
| | adult females | adult survival | acute | 48 h | | | | | NR | 2400 (2100-2700) | | | |
| ***Apocyclops borneoensis*** | adult females | reproduction (no. of nauplii /female) | chronic | 10 d | 30 | 7.9-8.25 | 20 | NR | tox values not calculated: 10 µg Ni/L reduced nauplii production by 33% | | | QA3 | Mohammed et al. (2010) |
| | | adult survival | acute | 48 h | | | | | NR | 13000 (11000-16000 | | | |
| ***Tigrious japonicus*** | adult females | reproduction (no. of nauplii /female) | chronic | 10 d | 30 | 7.9-8.25 | 20 | NR | tox values not calculated: 10 µg Ni/L reduced nauplii production by 32% | | | QA3 | Mohammed et al. (2010) |
| | | adult survival | acute | 48 h | | | | | NR | 18000 (13000-23000) | | | |
| **Mysids** | | | | | | | | | | | | | |
| ***Americamysis bahia*** [c] | post-larval stage | survival | acute | 96h | 25 ± 1 | NR | 30 ± 2 | <1-10 | NR | 150 | | QA1 | Lussier et al. (1999) |
| **Shrimps** | | | | | | | | | | | | | |

Table 2.3. Nickel toxicity data for tropical marine and estuarine crustaceans (rounded up to 2 significant figures). Grey shading indicates relevance to SEAM; no shading indicates species is not relevant to SEAM[a,b].

| | | | | | Water quality parameters | | | | Toxicity values (µg/L) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Life stage** | **Endpoint** | **Acute/ chronic** | **Test duration** | **Temp (ºC)** | **pH** | **Salinity (‰)** | **DOC (mg/L)** | **EC10** | **EC50** | **LOEC** | **Data quality** | **Reference** |
| ***Artemia urmiana* (brine shrimp)** | < 24h old nauplii | survival | acute | 24 h | 27 ± 1 | NR | 35 | NR | NR | 7.2 | | QA2 | Asadpour et al. (2013) |
| | | growth | chronic | 5, 11, 17 d | | | | | After 11-d growth reduced by ~50% at 3 µg Ni/L | | | | |
| | adult and nauplii | bioaccumulation | acute | 24 h | | | | | At 3 µg Ni/L concentration in nauplii = 0.03 µg/g, in adult = 0.035 µg/g | | | | |
| ***Artemia franciscana* (brine shrimp)** | < 24 h old nauplii | survival | acute | 24 h | 27 ± 1 | NR | 75 | NR | NR | 11 | | | |
| | | growth | chronic | 5, 11, 17 d | | | | | After 11-d growth reduced by ~63% at 3 µg Ni /L | | | | |
| | adult and nauplii | bioaccumulation | acute | 24 h | | | | | At 3 µg Ni/L concentration in nauplii = 0.015 µg/g, in adult = 0.055 µg/g | | | | |
| ***Artemia* [d #]** | embryos | emergence, development and survival | chronic | 72 h | 28 | NR | NR | NR | NR | >590 | | FAIL | MacRae and Pandey (1991) |
| | | | | | **Prawns** | | | | | | | | |
| ***Penaeus merguiensis* (banana prawn)[#]** | juvenile | survival | acute | 96 h | 20-35 | 8 ± 0.2 | 20, 36 | NR | NR | 3500 (1800-7100) | | FAIL | Denton and Burdon-Jones (1982) |
| ***Metapenaeus ensis* [#]** | larvae PZIII | survival | acute | 48 h | 27 ± 1 | 8.7 | NR - artificial seawater | NR | NR | 1300 | | FAIL | Wong et al. (1993) |
| | larvae MII | | | | | | | | NR | 9300 | | | |

Table 2.3. Nickel toxicity data for tropical marine and estuarine crustaceans (rounded up to 2 significant figures). Grey shading indicates relevance to SEAM; no shading indicates species is not relevant to SEAM[a,b].

| | | | | | Water quality parameters | | | | Toxicity values (µg/L) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Life stage** | **Endpoint** | **Acute/ chronic** | **Test duration** | **Temp (ºC)** | **pH** | **Salinity (‰)** | **DOC (mg/L)** | **EC10** | **EC50** | **LOEC** | **Data quality** | **Reference** |
| | PL3 postlarval stage | | | | | | | | NR | 8900 | | | |

[a] All toxicity values are measured, dissolved Ni, unless otherwise stated
[b] All tests used $NiCl_2$ or $NiCl_2.6H_2O$, unless otherwise stated
[c] Previously known as *Mysidopsis bahia*
[d] Tested $NiCl_2$ and $NiSO_4$
[#]Nominal concentrations
NR Not reported

Table 2.4. Nickel toxicity data for tropical marine molluscs (rounded up to 2 significant figures). Grey shading indicates relevance to SEAM; no shading indicates species is not relevant to SEAM[a, b].

| | | | | | Water quality parameters | | | | Toxicity values (µg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Life stage** | **Endpoint** | **Acute/ chronic** | **Test duration** | **Temp (°C)** | **pH** | **Salinity (‰)** | **DOC (mg/L)** | **EC10** | **EC50** | **Data quality** | **Reference** |
| **Bivalves** | | | | | | | | | | | | |
| ***Crassostrea virginica***[#] | embryos | survival | acute | 48 h | 25 ± 1 | NR | 24 ± 2 | NR | NR | 1200 | FAIL | Calabrese et al. (1977) |
| | larvae | survival | acute | 12 d | | | | | NR | 12000 | | |
| ***Tridacna maxima***[#] | fecundated egg | development to D larvae (veliger) | chronic | 48 h | 27 | 8.4 | 34 | NR | 100 | 110 | QA3 | AquaBiotech. Jocelyn Senia, Pers comm. |
| ***Saccostrea rhizophore***[#] | fecundated egg | development to D larvae (veliger) | chronic | 48 h | 27 | 8.4 | 34 | NR | 120 | 290 | QA3 | |
| ***Mimachlamys gloriosa***[#] | fecundated egg | development to D larvae (veliger) | chronic | 48 h | 24 | 8.4 | 34 | NR | 12 | 26 | QA3 | |
| ***Bractechlamys vexillum***[#] | fecundated egg | development to D larvae (veliger) | chronic | 48 h | 24 | 8.4 | 34 | NR | 12 | 13 | QA3 | |
| **Gastropods** | | | | | | | | | | | | |
| ***Babylonia areolata*** | adult | survival | acute | 96 h | 25 | ~8 | ~35 | ~8 | NR | 36000 (35000-28000) | QA2 | Hajimad and Vedamanikam (2013) |
| | larvae | survival | acute | 96 h | 25 | ~8 | ~35 | ~8 | NR | 200 (110-340) | QA2 | Vedamanikam and Hayimad (2013) |

[a] All toxicity values are measured, dissolved Ni, unless otherwise stated
[b] All tests used $NiCl_2$ or $NiCl_26H_2O$, unless otherwise stated
[#] Nominal concentrations
NR not reported

Table 2.5. Nickel toxicity data for tropical marine echinoderms and annelids (rounded up to 2 significant figures). Grey shading indicates relevance to SEAM; no shading indicates species is not relevant to SEAM[a,b].

| | | | | | Water quality parameters | | | | | Toxicity values (µg/L) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Life stage** | **Endpoint** | **Acute/ chronic** | **Test duration** | **Temp (°C)** | **pH** | **Salinity (‰)** | **DOC (mg/L)** | **EC10** | **EC50** | **NOEC** | **LOEC** | **Data quality** | **Reference** |
| **Echinoderm** | | | | | | | | | | | | | | |
| **Sea urchins** | | | | | | | | | | | | | | |
| ***Diadema savignyi*** | gametes | fertilisation and development | chronic | 48 h | 25 | 8.1 | 34 | 0.8 | | Test 1: 120 (100-140). Test 2: 72 (63-80) | 24 | 37 | QA1 | Rosen et al. (2015) |
| ***Diadema setosum*** | fecundated egg | development to pluteus | chronic | 48 h | 27 | 8.4 | 34 | NR | 50 | 75 | NR | NR | QA3 | Aqua Biotech. Jocelyn Senia, Pers comm. |
| ***Echinometra mathaei*** | fecundated egg | development to pluteus | chronic | 48 h | 27 | 8.4 | 34 | NR | 40 | 63 | NR | NR | QA3 | |
| **Annelid** | | | | | | | | | | | | | | |
| **Polychaetes** | | | | | | | | | | | | | | |
| ***Hydriodes elegans*** | gametes | sperm viability/ fertilization | chronic | 1 h | 28 ± 1 | 8.1 ± 0.1 | 34 ± 0.5 | NR | NR | 770 (590-1100) | NR | NR | QA1 | Gopalakrishnan et al. (2008) |
| | | egg viability/ fertilization | chronic | 1 h | | | | | | 1200 (790-2100) | | | | |
| | | embryo development | chronic | 2 h | | | | | | 2300 (1200-700) | | | | |
| | "ripe" worms, tube length 5 cm | larval release | chronic | 20 h | | | | | | 410 (330-520) | | | | |
| | | larval settlement | chronic | 96 h | | | | | | 160 (140-190) | | | | |
| | | adult survival | acute | 96 h | | | | | | 1500 (1200-900) | | | | |

[a] All toxicity values are measured, dissolved Ni, unless otherwise stated
[b] All tests used $NiCl_2$ or $NiCl_2.6H_2O$, unless otherwise stated

Table 2.6. Nickel toxicity data for tropical marine cnidarians (rounded up to 2 significant figures). Grey shading indicates relevance to SEAM; no shading indicates species is not relevant to SEAM[a,b].

| | | | | | Water quality parameters | | | | Toxicity values (µg/L) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Life stage** | **Endpoint** | **Acute/ chronic** | **Test duration** | **Temp (ºC)** | **pH** | **Salinity (‰)** | **DOC (mg/L)** | **EC10** | **EC50** | **NOEC** | **LOEC** | **Data quality** | **Reference** |
| **Anemones** | | | | | | | | | | | | | | |
| ***Aiptasia pulchella*** | juvenile (1-2 mm pedal disc diameter) | survival | acute | 24 h | 25± 1 | 7.90 - 8.40 | 35-40 | NR | NR | 4700 | NR | NR | QA1 | Howe et al. (2014a) |
| | | | | 48 h | | | | | | 3300 (3090-5050) | | | | |
| | | | | 96 h | | | | | | 2200 (1400-3800) | | | | |
| | | severe tentacle retraction | acute | 1 h | | | | NR | NR | 3500-5100 | NR | NR | QA1 | |
| | | | | 6 h | | | | | | 2300-2600 | | | | |
| | | | | 12 h | | | | | | 1400-3300 | | | | |
| | lacerate tentacle | development to juvenile | chronic | 8 d | 25± 1 | 8.0-8.4 | 34-35 | NR | NC | >490 | NR | NR | QA1 | Howe et al. (2014b) |
| | | | | 14 d | | | | | 260 (40-740) | >490 | | | | |
| | | survival | | 8 d | | | | | NC | NC | NC | NC | QA1 | |
| | | | | 14 d | | | | | NC | NC | NC | NC | | |
| | reproductive adult, pedal disc diameter of 3-4 mm | reproduction-total number of offspring | chronic | 28 d | 25± 1 | 8.20 - 8.50 | NR | NR | 260 (20-300) | 400 (310-420) | NR | 510 | QA1 | Howe et al. (2014c) |
| | | reproduction-total number juveniles | | | | | | | 65 (10-290) | 370 (220-410) | NR | 510 | | |
| **Corals** | | | | | | | | | | | | | | |

Table 2.6. Nickel toxicity data for tropical marine cnidarians (rounded up to 2 significant figures). Grey shading indicates relevance to SEAM; no shading indicates species is not relevant to SEAM[a,b].

| | | | | | Water quality parameters | | | | Toxicity values (µg/L) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Life stage** | **Endpoint** | **Acute/ chronic** | **Test duration** | **Temp (ºC)** | **pH** | **Salinity (‰)** | **DOC (mg/L)** | **EC10** | **EC50** | **NOEC** | **LOEC** | **Data quality** | **Reference** |
| ***Goniastrea aspera*** [c] | gametes | fertilisation success | chronic | 5 h | NR | NR | NR | NR | NR | >2000 | NR | NR | FAIL | Reichelt-Brushett and Harrison (2005) |
| ***Platygyra daedalea*** | gametes | fertilisation success | chronic | 5 h | NR | NR | NR | NR | NR | 1420 (1160-1800) | NR | NR | QA2 | Reichelt-Brushett and Hudspith (2016) |
| ***Pocillopora dmicornis*** [d] | planulae larvae | larval settlement | chronic | 12-96 h | 25-28 | NR | NR | NR | NR | NR | NR | NR | QA2 | Goh (1991) |
| | | survival | acute | 12 - 72 h | | | | | NR | 9000 | NR | NR | | |

[a] All toxicity values are measured, dissolved Ni, unless otherwise stated
[b] All tests used $NiCl_2$ or $NiCl_2.6H_2O$, unless otherwise stated
[c] Not included in SSD because a reliable toxicity value could not be calculated, and nominal Ni concentrations were used
[d] Toxicity value not included in SSD because EC50 was derived during recovery period, following exposure to nickel
NC not calculated
NR not reported

Table 2.7. Nickel toxicity data for tropical marine and estuarine fish (rounded up to 2 significant figures). Grey shading indicates relevance to SEAM; no shading indicates species is not relevant to SEAM[a]. All values reported in this table used nominal concentrations.

| | | | | | Water quality parameters | | | | Toxicity values (µg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Life stage** | **Endpoint** | **Acute/ chronic** | **Test duration** | **Temp (°C)** | **pH** | **Salinity (‰)** | **DOC (mg/L)** | **EC10** | **EC50** | **Data quality** | **Reference** |
| ***Priopidichthys marianus*** | juvenile | survival | acute | 96 h | 30 | NR | 36 | NR | NR | 100000 (80000-125000) | FAIL | Denton and Burdon-Jones (1986) |
| ***Liza klunzingeri*** | NR | survival | acute | 71 h | 25± 2 | 8.2 | NR | NR | NR | 4.2 (3.2-5) | FAIL | Bu-olayan and Thomas (2005) |
| ***Leiostomus xanthurus*** | adult | survival | acute | 96 h | 25-26 | NR | 21 | NR | NR | 70000 (57000-88000) | FAIL | USEPA ecotox database. Ref 3732 |
| ***Menidia peninsulae*** | NR | survival | acute | 96 h | 25 | NR | 20 | NR | NR | 38000 (30000-45000) | FAIL | |

[a] All tests used $NiCl_2$ or $NiCl_2.6H_2O$, unless otherwise stated
NR not reported

### *Species sensitivity distributions*

There were only a limited number of high-quality data for tropical marine and SEAM-relevant species that could be used in the SSDs. Using only QA1 data, there were six and five data for tropical and SEAM relevant species, respectively (Figure 2.2 A and B). The inclusion of all data scored as QA1, QA2 or QA3 added eight additional data points to both the tropical and SEAM SSDs (Figure 2.2 C and D). These were the coral (QA2) and the copepod, oyster, giant clam, scallop, seashell and two additional sea urchin data (QA3). The euryhaline copepod *Acartia pacifica* was included in the marine SSD, although this test was conducted at 25‰, because this species is known to have a coastal distribution. Given that an increase in salinity is usually considered to be protective against nickel toxicity (Blewett et al., 2015), this value may be conservative for marine systems. When all data are included in the SSDs, there are a sufficient number of data to derive a protective concentration value (i.e. >10 data in Figure 2.2 C and D). However, these SSDs include data which were not scored of high quality; specifically in some cases, the nickel concentrations in the test solutions were not measured. Based on the SSDs using high quality data (Figure 2.2 A and B), there is insufficient data to derive a reliable protective concentration value (guideline value).

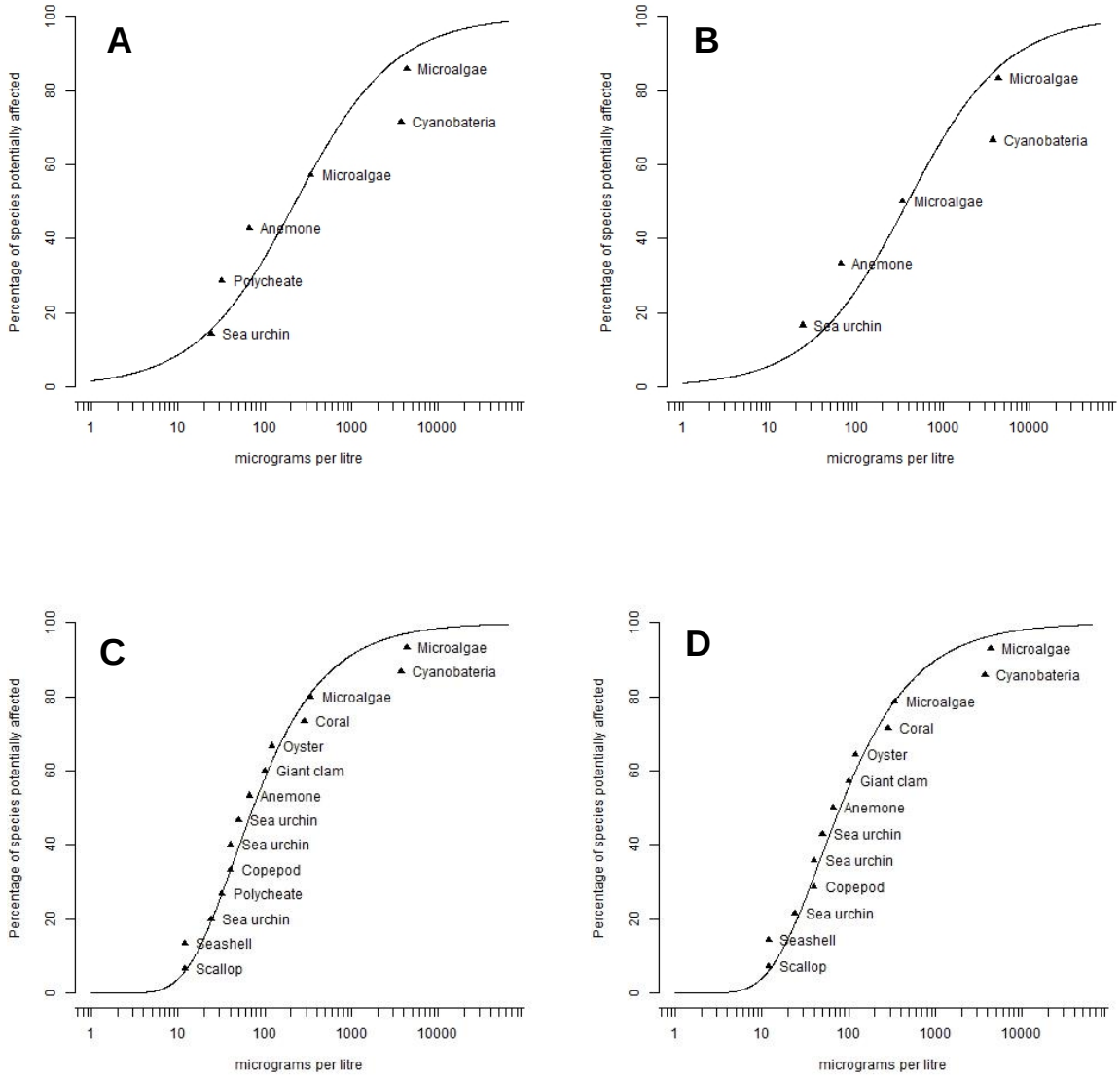



Figure 2.2. Species sensitivity distributions using chronic nickel toxicity data for high quality QA1 data for (A) all tropical marine species and (B) species relevant to SEAM, and for all QA1, QA2 and QA3 data for (C) all tropical marine species and (D) species relevant to SEAM. All data were scored based on the data quality criteria checklist (Table 2.1).

### 2.3.2. Tropical marine toxicity tests

Given the absence of sufficient quality nickel toxicity data for tropical species to derive a nickel WQG, the next step was to determine if new data could be generated using existing tropical bioassays. These toxicity tests are presented in Appendix A (Tables A1-9). Of the SEAM-relevant species, two microalgae tests, three copepod tests, one barnacle test, four bivalve tests, one gastropod test, two sea urchin tests, one anemone test, four coral tests and one fish test were confirmed to be available for use. Toxicity tests existed for crabs, prawns, star fish and fish, however further investigation was required to identify whether or not these species were still available for routine use. Most tests were chronic, although some acute tests (acute copepod and fish survival) were identified.

Further tropical test development is required for several taxonomic groups including macroalgae, seagrass, mangroves, and fish. In particular, there were no available chronic fish tests with tropical or SEAM species, although studies with temperate biota have suggested that fish are relatively insensitive to nickel. For many taxonomic groups there were only one or two test species available that are relevant to SEAM, for example, gastropods and anemones, both of which were sensitive to nickel, therefore test development with additional species would be useful.

### 2.3.3. Gap analysis

Once nickel toxicity data were compiled and scored, gaps were identified, based on the existing taxonomic spread of data (particularly with relevance to WQG requirements of a minimum of eight, and preferred 15 species across four taxonomic groups); sensitivity of particular taxa from other nickel studies (e.g. temperate studies, acute tropical studies); availability of high quality chronic data; and ecological relevance of taxa in SEAM. Appendix A, Table A10 provides a review on the diversity and relevance of certain taxa to SEAM and this was used to justify and prioritise further testing with key taxa.

*Filling the data gaps*

Ideally, to generate a reliable guideline value for nickel in marine waters of SEAM, high quality data (QA1) from ≥ eight species from ≥ four taxonomic groups are required. There were only five data (five different species) scored as QA1, from only three taxonomic groups (Figure 2.2 B), therefore further research is needed to fill the gaps in nickel toxicity data for SEAM.

As shown in Section 2.3.1, high quality chronic data exist for one species of anemone and one sea urchin, both relevant to SEAM. Chronic low-quality data exists for SEAM-relevant bivalves and echinoderms. Only low-quality acute data were available for one gastropod species found in SEAM. At the time this review was written, there were no known high-quality nickel toxicity data available for corals, mangroves, macroalgae, seagrasses, crustaceans, molluscs, ascidians or fish.

Key structural habitats in SEAM are coral reefs, mangroves and seagrasses, all of which are critical to protect ecosystem services such as biodiversity. These habitat types are connected through many species of fish and invertebrates that use them as breeding grounds and/or nurseries but may live as adults in another habitat. In particular, many reef species rely on adjacent mangrove and seagrass habitats. Impacts in one of these habitats could indirectly impact other habitats. Important species relevant to these ecosystems include not only corals, mangroves and seagrasses, but other taxa such as crustaceans, molluscs (bivalves, gastropods and cephalopods), echinoderms (sea urchins, star fish, sea cucumbers), other cnidarians (anemones), ascidians and fish.

Mangroves act as a buffer zone in estuaries protecting seagrass meadows and coral reefs; they are also key structural habitats in tropical estuaries and act as breeding grounds and nurseries for fishes and invertebrates. Mangroves provide high productivity, abundant detritus and high throughputs of organic carbon, although the latter are often subject to very high rates of microbial consumption in tropical estuaries (e.g. Robertson et al., (1991, 1990)).

Seagrasses are highly productive systems that support unique faunal assemblages from epiphytic invertebrates to herds of grazing dugong. Toxicity testing with tropical mangroves and seagrasses is not yet well established and further research and development is required. Although mangroves and seagrasses are important tropical habitats, there are no laboratory-based toxicity tests currently available for use. Any further test development will likely be based on *in situ* measurement of photosynthesis in seagrasses, but such endpoints are not routinely used in deriving guideline values.

Coral reefs provide fundamental habitats in tropical systems and are the most diverse of all marine ecosystems, reaching their pinnacle of biodiversity in the region known as the coral triangle between the Philippines, Indonesia and Papua New Guinea. Anemones, in the same class as corals have been shown to be sensitive to nickel (Table 2.6). At the time of this data compilation and gap analysis, three studies were found which investigated the effects of nickel on coral fertilisation and larval settlement and survival. Based on fertilisation success, effects of nickel on fertilisation in two species of corals were observed between 1420 and >2000 µg Ni/L (Reichelt-Brushett and Harrison, 2005; Reichelt-Brushett and Hudspith, 2016). However, these data were not scored of high quality (Table 2.6). Another study found larval survival to be inhibited at 9000 µg Ni/L; this data was generated from recovery time, following exposure to nickel, and was scored as QA2 in the quality assessment (Table 2.6) (Goh, 1991). Based on the ecological importance of corals to tropical marine systems and the limited nickel toxicity data currently available, it is recommended that investigating nickel toxicity to coral species is a high priority. Toxicity tests are available for corals relevant to SEAM.

Macroalgae are not considered to be the main habitat providers in tropical systems; they are not as dominant and have a lower species diversity than in cooler temperate regions (Mejia et al., 2012). Nonetheless, macroalgae are still ecologically important species, particularly on coral reefs where crustose coralline algae play a significant role in limestone formation and consolidation of loose substrates providing a surface for coral larvae to settle (Mejia et al.,

2012). Macroalgae also play a key role in nitrogen fixation and as a food source for higher trophic organisms (Chaves et al., 2013; Diaz-Pulido and McCook, 2008). Nickel data and toxicity tests are not available for tropical marine macroalgae. As for seagrasses and mangroves, toxicity testing is not well established for tropical macroalgae.

Anemones are important species found in coral reefs of SEAM and are widely distributed throughout tropical and subtropical ecosystems (Howe et al., 2014b). Sea urchins are also important inhabitants of coral reefs; high diversity of sea urchins has been recorded in the Caribbean, and, while there are limited sampling sites in SEAM, asteroids (starfish) and holothurians (sea cucumbers) appear to dominate in the Northeast Pacific (Iken et al., 2010). Mollusc diversity (bivalves and gastropods) is highest in the tropics, particularly in the Indo-Pacific coral reef environments (Bouchet et al., 2002). High numbers of bivalves have been found in New Caledonia and studies have shown that gastropod diversity is also highest in New Guinea, Indonesia and the Philippines (Bouchet et al., 2002; Roberts et al., 2002; Wells, 1990). Gastropods and bivalves are also an important food source for the tropical island nations of SEAM. From the data presented in Section 2.3.1, anemones, sea urchins and bivalves are the most sensitive species to nickel, with toxic effects observed between 12 and 120 µg Ni/L (Table 2.4 –Table 2.6). The acute data available for one gastropod species indicate that juveniles are more sensitive to nickel than adults (Table 2.4). One freshwater study also showed that a tropical snail was highly sensitive to nickel, with chronic juvenile growth inhibited by 20% (EC20) at 1.3 µg Ni/L (Niyogi et al., 2014). Based on the sensitivity to nickel and importance of cnidarians (corals and anemones), echinoderms and molluscs to SEAM, these taxa are classified as a high priority for further testing.

Crustaceans, including copepods, amphipods, barnacles, shrimps and lobsters have high species diversity in the Indo-Pacific, particularly living in association with coral reefs (Humes, 1994; Thomas, 1993; Williams et al., 1988). Mysids and shrimps (not relevant to SEAM) are sensitive to nickel, with acute mortality observed between 7 – 152 µg Ni/L (Section 2.3.1, Table 2.3). Chronic data was available for one estuarine copepod relevant to SEAM; after a

10-d exposure the LOEC value was 100 µg Ni/L. These few studies suggest that crustaceans may be sensitive to nickel. Despite their importance in tropical systems, few data on the sensitivity of these taxa to nickel were found.

Other important invertebrate taxa in tropical marine environments include cephalopods, sponges and ascidians. There is limited information on cephalopod diversity in SEAM, however one species of *Nautilus* is endemic to New Caledonia (Bustamante et al., 2000). Cephalopods are mostly large pelagic organisms and toxicity testing with such species is difficult. Likewise for sponges, there is limited information on their diversity or ecological importance in SEAM, although high regional diversity has been found in the Caribbean and has been related to their adaptability to a variety of habitats and the warm waters (Wulff, 2005). Sponges are filter feeders and could potentially be exposed to dissolved nickel. They also provide microhabitats for all trophic levels from bacteria to shrimp and fish (Hoeksema, 2007). Ascidians are sedentary filter feeders and have high diversity in tropical warm waters, particularly on the coral reefs of New Caledonia (Monniot et al., 1994). Currently, no toxicity test protocols exist for cephalopods, sponges or ascidians. Although their ecological importance has been demonstrated, further research is required to assess their suitability as toxicity test species. It is likely, particularly for cephalopods that this will be difficult, and so they have been classed as low-medium priority.

The richest fish diversity is found around Indonesia, New Guinea and the Philippines (Randall, 1998). Fish are important components of food webs in coral reefs, seagrasses and mangroves and form a link between these three habitats; some species of fish may live as adults in one habitat but use others as breeding grounds or nurseries (Nemeth, 2009). In the West Pacific Plate bioregion, encompassing the eastern part of SEAM, amphidromous fish and shrimp are common (e.g. many gobies, gudgeons, shrimps and prawns of the Families Atyidae and Palaemonidae and a number of freshwater crabs, particularly in the Family Grapsidae). They are born in freshwater, drift into coastal waters as larvae to develop in coastal and oceanic environments for a period of one month or more, and then migrate

back into freshwater to finish developing into full adults to spawn (e.g. see Keith et al., 2008). As a result, early life-stages of these organisms are faced with osmoregulatory dynamics as well as potential exposure to anthropogenic stressors as they migrate through estuaries and metamorphose. This combination of stressors may make the early life stages of these organisms particularly vulnerable to nickel exposure. Similarly, catadromous species (which live in freshwater but spawn in marine or estuarine environments, e.g. eels (Anguillidae), barramundi (Centropomidae), flagtails (Kuhlidae), and some grunters (Terapontidae)) and facultative freshwater species (marine species that are able to make use of freshwater habitats, e.g. mullet (Mugilidae), silverbiddies (Gerreidae), ponyfishes (Leiognathidae), croakers (Sciaenidae), and some perchlets (Ambassidae)) are also important components of freshwater ecosystems in this region, particularly east of Wallace's Line. These too will need to transition between marine and freshwater environments and adjust to osmotic differences as they pass through estuaries. Toxicity tests with amphidromas and catadromas species are challenging and so this remains a gap.

Coral reef fish are an important part of the economy as a food source and in tourism and recreational activities (Hoeksema, 2007). Although the ecological importance of fish to marine systems in SEAM is obvious, they have not been shown to be sensitive to nickel in past studies in tropical regions (Table 2.7.), and so development of chronic fish tests with SEAM-relevant species is not considered a high priority.

## 2.4. Conclusion

To manage potential impacts from the development of lateritic nickel ores in the region of SEAM, bioavailability-based predictive models and toxicity tests with endemic species are required to develop ecologically-relevant WQGs for nickel.

This data compilation and gap analysis identified that the most sensitive tropical marine species to nickel were echinoderms (sea urchins), anemones, crustaceans (copepod, mysid, and shrimp), gastropod (snail) and polychaetes, whereas corals and microalgae were less sensitive, however, only limited data are available for corals. Overall, very few high-quality

chronic nickel data were available for tropical marine species, and even less so for those relevant to SEAM. While corals, seagrasses and mangroves form key structural habitats in SEAM, toxicity tests are only likely to be available for corals.

Based on their ecological importance to SEAM, sensitivity to nickel, and to meet the data quality requirements for water quality guideline development, it is recommended that high quality chronic nickel data are acquired for, in order of priority: cnidarians (corals and anemones), molluscs (gastropods, bivalves), crustaceans (copepods, amphipods, barnacles, shrimps, prawns), echinoderms (sea urchins, star fish and sea cucumbers), macroalgae and fish.

## 2.5. Aims and objectives for this thesis

The overall aim of this project is to address these knowledge gaps and to improve our understanding of the environmental effects of waterborne exposure to nickel in the tropical marine ecosystems of the SEAM region.

Key objectives were to:

1. Develop and apply toxicity tests with relevant and unique species to the SEAM region to generate high quality nickel toxicity data; and
2. Incorporate high quality chronic nickel toxicity data into species sensitivity distributions to generate protective concentration values.

These objectives were addressed in the following chapters:

- Chapter 3 aimed to investigate the toxicity of nickel to tropical marine microalgae and *Symbiodinium* isolated from tropical marine hosts including corals.
- Chapter 4 elucidated the effect of nickel on two crustaceans (barnacle and copepod) and a gastropod (snail).
- Chapter 5 aimed to determine the toxicity of nickel to coral fertilisation success.

- Chapter 6 studied the effect of dissolved nickel exposures to adult corals and their microbiome (bacteria and *Symbiodinium*).
- Chapter 7 aimed to investigate the effect of dissolved and particulate nickel exposures on adult corals and their microbiome.
- Chapter 8 compiled existing data and new data acquired through this study into species sensitivity distributions to generate protective concentration values.

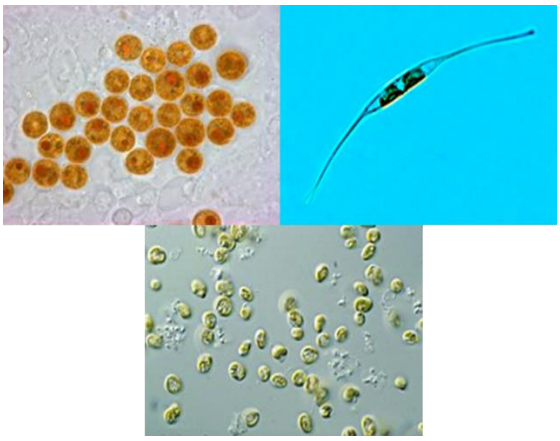
Ultimately, this research provides high quality, chronic nickel toxicity data which will contribute to the development of an ecologically-relevant WQG for nickel in tropical marine waters. In addition, copper, a commonly-used reference toxicant, was tested for quality assurance purposes, enabling comparisons with previous studies. Copper is a widespread contaminant in marine environments and toxic effects on marine organisms are frequently observed at environmentally realistic concentrations.

Research on the effects of nickel exposure on corals and their microbiome will provide valuable information on how corals, key structural habitats in tropical systems, respond to contaminant exposure. Results from the microbiome study will provide further insights into the complex relationship between corals and their microbiomes and the role the microbiome plays in dealing with contaminant stress.

# 3. TOXICITY OF NICKEL TO MARINE MICROALGAE

## Context statement

Given the crucial role that microalgae play in tropical marine environments, it is important to ensure this trophic level is represented in water quality guideline (WQG) development. This chapter builds on toxicity data for two microalgal species originally presented in Chapter 2. Initial investigations provided preliminary data on the toxicity of nickel to *Nitzschia closterium* (now known as *Ceratoneis closterium*) and *Isochrysis* sp. (now known as *Tisochrysis lutea*). This chapter reports on the toxicity of copper and nickel to three species of tropical marine microalgae; *Ceratoneis closterium*, from the class Bacillariophyceae, *Tisochrysis lutea* from the class Coccolithophyceae and *Symbiodinium sp Freud.Clade* C from the class Dinophyceae. This work, in combination with investigations on the effects of copper and nickel to tropical freshwater microalgae has been prepared for publication[2].



[2] McKnight, K., Gissi, F., Adams, M., Stone, S., Stauber, J., Jolley, D. Investigating the effects of nickel and copper on tropical marine and freshwater microalgae, using single and multispecies bioassays (in prep).
I completed toxicity tests with *Tisochrysis lutea* and *Symbiodinium.* Kitty McKnight completed toxicity tests with *Ceratoneis closterium.* Metal and statistical analysis of data was done by both myself and Kitty McKnight. I drafted this chapter. The data presented in this chapter has been included in the above-mentioned publication written by myself and Kitty McKnight with input from co-authors.

## 3.1. Introduction

Microalgae, as photosynthetic organisms play a vital role in aquatic ecosystems; they are the primary link of aquatic food chains and sensitive indicators of environmental change (Kuzminov et al., 2013; Levy et al., 2007; Rasdi & Qin, 2015). Additionally, microalgae are easy to culture and are amenable to laboratory testing (Franklin et al., 2005). Therefore, microalgae are important test species to include in regulatory assessment of metals (Levy et al., 2007).

The sensitivity of microalgae to metals varies between species (Levy et al., 2007). Microalgae of the classes Dinophyceae, Bacillariophyceae and Coccolithophyceae play important roles in tropical marine ecosystems. *Symbiodinium* of the class Dinophyceae, are known for their important role on coral reefs. Reef-building corals form symbiotic relationships with trophic dinoflagellates in the genus *Symbiodinium*, which contribute over 95% of organic nutrients and the carbon requirements of the coral host, and also enhances calcification rates (Boulotte et al., 2016; Kuzminov et al., 2013). Microalgae of the class Bacillariophyceae, or more commonly diatoms, are ecologically important as they contribute 20% of the global primary production and oceanic biogeochemical cycling (Hook et al., 2014). Diatoms such as *Ceratoneis closterium* have been widely studied and there is sufficient toxicity data available in the literature with which to make comparisons (Florence et al., 1994; Levy et al., 2007; Stauber and Florence, 1987). Microalgae of the class Coccolithophyceae, such as *Tisochrysis lutea*, are high in fatty acids and an important food source for higher trophic organisms both in the natural environment and in aquaculture (Rasdi and Qin, 2015).

This chapter aims to address the first objective outlined in Chapter 2 and has investigated the sensitivities of a range of tropical marine microalgae to nickel and copper using chronic 72-h growth rate inhibition bioassays. Three different species were used from three classes: Dinophyceae, Bacillariophyceae and Coccolithophyceae. Initial investigations provided preliminary data on the toxicity of nickel to *Nitzschia closterium* (now known as *Ceratoneis*

*closterium*) and *Isochrysis* sp. (now known as *Tisochrysis lutea*) (Chapter 2). These initial investigations found that these species were relatively insensitive to nickel, however, there are limited high quality data available in the literature on the toxicity of nickel to tropical marine microalgae. Additional definitive toxicity tests were conducted with copper and nickel with both these species, together with a dinoflagellate, isolated from a coral host, *Symbiodinium* sp Freud.Clade C. In this and subsequent chapters, copper has been included as a reference toxicant to allow for comparison with previous studies. There is substantially more copper toxicity data available in the literature with which to compare results, most likely due to the ubiquity and high toxicity of copper in marine environments (Levy et al., 2007).

## 3.2. Methods

### 3.2.1. General laboratory techniques and reagents

All glassware and plastic containers used throughout the study were acid-washed in 10% (v/v) nitric acid (Reagent grade Merck, Darmstadt, Germany) and thoroughly rinsed with demineralised water (five rinses), followed by high purity water (5 rinses) (Milli-Q®, 18.2MΩ/ cm; Merck).

Growth rate inhibition tests were conducted in borosilicate glass Erlenmeyer flasks which were pretreated with Coatasil (Ajax Finechem) to prevent adsorption of metals to the glass. Flasks were then soaked in 10% nitric acid (Reagent grade Merck) for 24 h and rinsed thoroughly with high purity water.

All metal stock solutions were made volumetrically using high purity water. Copper stock solutions of 0.005 and 0.1 g Cu/L were prepared using copper (II) sulfate salt (A.R. grade, AJAX Chemicals, Australia), and acidified to 0.1% HCl (Tracepur, Merck). Nickel stock solutions of 0.010, 0.1, 1 and 8 g Ni/L were made using nickel (II) chloride hexahydrate salt (AR grade, Chem Supply, Australia) and acidified to 0.1% HCl (Tracepur, Merck).

Seawater used for culturing and in toxicity tests was collected from Cronulla, NSW, Australia, in high-density polyethylene (HDPE) containers, filtered through an acid-washed (10% v/v, nitric acid, Merck) 0.45 µm cartridge filter (Sartobran P sterile midicap, Sartorius Stedium Biotech, Germany), and stored in polyethylene containers at 4°C in the dark.

The physico-chemical parameters of the seawater used in toxicity tests were measured prior to test commencement. The pH of each treatment solution was measured on Day 0 and Day 3 using one replicate flask from each treatment. Salinity measurements were taken using a YSI salinity and conductivity meter (model30/10FT, YSI, Ohio, USA). The pH was measured using a Thermo Orion pH meter with an epoxy body probe (meter model 420, probe model ROSS815600, Thermo Fisher Scientific, USA) which was calibrated daily. Dissolved oxygen (%) was measured using an Oximeter (Oxi330WTW, Weilheim, Germany), which was calibrated immediately prior to use.

### 3.2.2. Culturing

This study used four different strains of microalgae across three classes, Dinophyceae, Bacillariophyceae and Coccolithophyceae. All microalgal species were originally obtained from the CSIRO Collection of Living Microalgae, Marine and Atmospheric Research Hobart, Australia. All species were cultured in controlled light (70 µmol PAR photons/$m^2$/s, on a 12:12 h light: dark cycle, cool while) at 27 ± 2°C in temperature controlled rooms. All species were cultured under axenic conditions. Descriptions of the species are provided in Table 3.1.

Table 3.1. Culture conditions for tropical marine microalgae

| Algal species | Origin of isolate | Culture medium |
|---|---|---|
| **Dinophyceae** | | |
| *Symbiodinium* sp Freud.Clade C | Coral host *Montipora verrucosa* (from Hawaii, USA) | F/2[a,b] |
| **Bacillariophyceae** | | |
| *Ceratoneis closterium* | Coral Sea | F/2[a] |
| *Ceratoneis closterium* | Coral Sea | G/2[c] |
| **Coccolithophyceae** | | |
| *Tisochrysis lutea* | Tahiti | F/2[a] |

[a] Guillard and Ryther, 1962
[b] 2 x Fe stock added
[c] Loeblich and Smith, 1968

### 3.2.3. Growth rate inhibition tests

Toxicity test methods followed Franklin *et al*. (2005) and OECD Test Guidelines (OECD 2011b). Test conditions are outlined in Table 3.2. Briefly, test solutions were supplemented with 1.5 mg $NO_3^-$/L (as sodium nitrate, BDR AR Grade) and 0.15 mg $PO_3^{4-}$/L (as potassium dihydrogen phosphate, BDR AR Grade) to sustain exponential algal growth throughout the 72-h exposure. Flasks were spiked with either copper or nickel. For nickel, nominal concentrations ranged from 20 – 1730 µg Ni/L, 10 – 43250 µg Ni/L, 1000 – 57 665 µg Ni/L and 50 – 14600 µg Ni/L for *Symbiodinium*, *C. closterium* (F2), *C. closterium* (G2), and *T. lutea*, respectively. For copper tests nominal concentrations ranged from 2 – 12 µg Cu/L, 1 - 40 µg Cu/L, 0.25 – 20 µg Cu/L, and 0.5 – 16 µg Cu/L for *Symbiodinium*, *C. closterium* (F2), *C. closterium* (G2), and *T. lutea*, respectively. The full range of nominal concentrations for each test are provided in Appendix B (Tables B3-4).

Cultures of algae (in exponential growth phase, 6-7 days) were centrifuged at 280 g for 7 minutes and washed four times to remove nutrient rich culture media, except for *Symbiodinium*, which was not washed. Initial investigations found that the growth rate of *Symbiodinium* was significantly reduced after washing and centrifugation. Concentrated algae were vortexed (*T. lutea*) or homogenised (*C. closterium)* in a hand-held homogeniser (15 mL, Wheaton), to re-suspend the algal cells. Cell densities were calculated using flow cytometry (FACSCalibur, BD Bioscience) and each flask was inoculated with 1-3 $x10^3$ cells/mL and incubated in a temperature and light controlled cabinet (Table 3.2). These low initial cell densities were used to better emulate cell densities in the natural environment and to reduce any potential metal speciation changes over the duration of the test (Franklin et al., 2002).

Cell densities were determined at 24, 48 and 72 h, using flow cytometry (FACSCalibur, BD Sciences). Cells were excited at 488 nm and densities of algae were measured using chlorophyll *a* autofluorescence and side angle light scatter. In all tests, 0.5 mL was sub-sampled from each flask and 0.15 mL of fluorescent beads (BD Trucount™Tubes, BD

Biosciences) were added as an internal counting standard (Franklin et al., 2005). In bioassays with *Symbiodiunium* and *C. closterium,* flasks were gently scrapped using disposable Pasteur pipettes to loosen the clumped cells attached to the glass. Aliquots from each flask, were then homogenized in a hand-held homogenizer (15 mL, Wheaton) prior to sampling. For *T. lutea,* the flasks were swirled to homogenise the solution prior to subsampling.

Growth rate (cell division μ) was calculated as the slope of the regression line from a plot of $\log_{10}$ (cell density) versus time (h). Growth rates for each treatment flask were converted to the percent of control (Levy et al., 2007). Three definitive tests were conducted with *Symbiodinium, T. lutea* and *C. closterium* (G2). One range finder and one definitive test were conducted for *C. closterium* (F2). Additional definitive tests with *C. closterium* (F2) were not completed because it was shown that the response of *C. closterium* (F2) to copper and nickel was similar to *C. closterium* (G2). For each species, data from all definitive tests were pooled for statistical analysis. Two replicates per treatment were chosen to allow for testing of a wider concentration range that encapsulated the range of sensitivities of the microalgae.

Table 3.2. Toxicity test conditions for 72-h growth rate inhibition tests with microalgae

| **Toxicity test parameters** | |
|---|---|
| Temperature (°C) | 27 ± 1 |
| pH | 8.1 ± 0.2 |
| Salinity (‰) | 35 ± 1 |
| Conductivity (mS/cm) | 54 ± 1 |
| Dissolved oxygen (%) | >90 |
| Light | Cool white fluorescent lighting, 140 μmol $m^2$/s |
| Photoperiod | 12:12 h Light: dark |
| Test type | Static no renewal |
| Test duration | 72 h |
| Test chamber | 250 mL glass flasks |
| Test solution volume | 50 mL |
| Age of test organism | Exponential growth phase<br>5- 6 days old - *C. closterium* and *T. lutea*<br>7-9 days old - *Symbiodinium* |
| Initial cell density | 1-3 x$10^3$ cells/mL |
| No. of control replicates | 4 |
| No. of treatment replicates | 2 |
| Control/diluent water | 0.45-μm filtered seawater |
| Test endpoint | Growth rate (cell division rate) |
| Test acceptability | Growth rate in controls: 2.2 ± 0.3[a] (*C. closterium* and *T. lutea*) 1 ± 0.3[a] (*Symbiodinium*) doublings/day, <1 unit change in pH |

[a] Results based on internal QA database, mean ± 2 x standard deviation

### 3.2.4. Chemical analyses

All plastic-ware used for metal sub-sampling was acid washed (10% v/v, Tracepur; Merck) and rinsed with high purity water in a semi-clean room. All dissolved metal sub-samples were filtered through acid-washed syringes and 0.45-µm sterile filters (Sartorius Ministart® Syringe Filter, Germany), collected in acid-washed 5-mL or 10-mL polypropylene vials and acidified to either 0.2% (dissolved metals) or 2% (total metals) with Tracepur nitric acid (Merck). Sub-samples for metal analyses were taken from the test flasks on Day 0 (after the addition of microalgae) and Day 3 (test completion).

Most samples were analysed using inductively coupled plasma-atomic emission spectrometry (ICP-AES 730ES), except for copper samples which were below, or close to the detection limit of the ICP-AES (~1 µg Cu/L). Instead, these samples were analysed by ICP–mass spectrometry (ICP-MS; Agilent 7500CE), with a detection limit of ~0.1 µg Cu/L. Quality assurance procedures included matrix-matched calibration standards, drift standards and seawater blanks.

Sub-samples were taken from the seawater used in all tests to measure dissolved organic carbon (DOC) at the beginning of each test. Samples were filtered through a 0.45-µm filter and collected in a glass vial with 2 mL of concentrated $H_2SO_4$. Analysis of DOC was carried out at the National Measurement Institute (NMI), Sydney, Australia.

### 3.2.5. Statistical analysis

The average (T= 0 and 72 h) measured dissolved metal concentrations were used in the statistical analyses. For each species and metal, data from all replicate tests were combined and analysed using the free software R (version 3.3.2, 2016-10-31) (R Core Team, 2016) in the drc package (version 3.0-1, (Ritz et al., 2015)). For each dataset, 3-4 different models including the log-logistic models with 3 and 4 parameters, LL.3 and LL.4, and the Weibull models with 3 parameters (W1.3, W2.3) were fitted to the concentration-response curves. The model of best fit was selected using the Akaike information criterion (AIC, i.e. lowest AIC value, Appendix B, Table B1), and by visual assessment of the model. The chosen model

was then used to determine toxicity estimates (Effect Concentration to cause x% effect relative to the control, ECx). The GGplot package was used to generate a graphical representation of the data and the model used to calculate toxicity estimates.

Bonferroni's t-test was used to determine which treatments were significantly different from the control (2-tailed, $p<0.05$) to determine the no-observed-effect concentration (NOEC) and the lowest-observed-effect concentration (LOEC) using Toxcalc (Ver 5.0.23; TidePool Scientific Software). While the scientific community is moving away from the use of NOECs and LOECs, these values have been included in this and subsequent chapters to allow for comparisons with previous studies where NOEC and LOEC values were used. In addition, it was not always possible to calculate reliable toxicity estimates (effect concentration values).

## 3.3. Results

### 3.3.1. Quality control

In all tests, over the 72-h exposure period, physico-chemical parameters were within acceptable limits (Table 3.2), measured values of: pH 8.0 ± 0.1, salinity 35 ± 0.6 ‰, conductivity 54 ± 1.1 mS/cm, DO 105 ± 5% (± standard deviation, SD). Temperature was maintained at 27 ± 1°C. The mean DOC concentration (± SD) in seawater used in the toxicity tests was 0.9 ± 0.2 mg/L (n = 10, data not shown).

Background concentrations of dissolved metals in seawater used in all tests were generally below the limit of detection (LOD µg/L, Al 0.42, As 2.9, Ba 0.04, Cd 0.51, Co 0.42, Cr 0.48, Cu 0.72, Fe 0.76, Mn 0.06, Ni 0.96, Pb 2.3, Se 4.3, Zn 0.19). In tests with *C. closterium* and *T. lutea*, some dissolved metals in the seawater exceeded their LODs including As (3 µg/L), Ba (4 µg/L), Cr (0.53 µg/L), Fe (5.7 µg/L), Mn (0.18 µg/L), Pb (4.1 µg/L), and Zn (1.7 µg/L) (Appendix B, Table B2).

In all tests, the measured dissolved metal concentrations were between 78-100% for nickel and 37-100% for copper, of the nominal values. Across all toxicity tests, the loss in dissolved metal over 72 h ranged from 0 – 19% for nickel and from 3 – 100% for copper (Tables B3,

B4). The greatest copper loss was observed in toxicity tests with *T. lutea* at the lowest nominal concentrations of 0.5 and 1 µg Cu/L (94-100% loss in copper). The loss of copper in higher test concentrations with this species (2 – 16 µg Cu/L, nominal) was far less, between 4 – 48%. For tests with *C. closterium* (F2 and G2), copper loss ranged between 12 – 64%; for *Symbiodinium,* copper loss ranged from 3 – 50% (Appendix B, Tables B3, B4).

Across all definitive tests for each species, the control growth rate met the acceptability criteria. For *C. closterium* (G2) the mean control growth rate (doublings/day ± 1 SD) was 2.2 ± 0.01, for *C. closterium* (F2) it was 2.1 ± 0.04, for *T. lutea* it was 2.4 ± 0.07 and for *Symbiodinium* it was 0.9 ± 0.1. Across all tests the unit change in pH over 72 h was <0.6 (data not shown).

### 3.3.2. Toxicity of nickel to tropical marine microalgae

Tropical marine microalgae from different classes responded differently to nickel exposure (Figure 3.1. A). From the concentration-response curves (Figure 3.1. A) it is evident that *T. lutea* and *Symbiodinium* sp. are more sensitive to nickel than both strains of *C. closterium*. Growth rate was inhibited by 10% (EC10) from 330 µg Ni/L for *T. lutea* to 3250 µg Ni/L for *C. closterium* (G2). Reliable EC10 estimates could not be calculated for *Symbiodinium*. Based on no observable effect concentration (NOEC) values, the most to least sensitive algae in this study were *T. lutea*, *Symbiodinium* and *C. closterium* (Table 3.3). There was little difference in the sensitivity of *C. closterium* to nickel when cultured in G2 or F2 medium (Table 3.3, Figure 3.1 A).

### 3.3.3. Toxicity of copper to tropical marine microalgae

Tropical marine microalgae were found to be more sensitive to copper than nickel (Figure 3.1B,Table 3.3). The concentration-response curve in Figure 3.1 B showed the reverse for copper compared to nickel with the diatom, *C. closterium* more sensitive to lower concentrations of copper compared to *T. lutea* and *Symbiodinium* sp. Based on EC10 values, *C. closterium* (0.87 – 0.97 µg Cu/L) was most sensitive, followed by *T. lutea* (2.2 µg Cu/L) and then *Symbiodinium* (3.3 µg Cu/L) (Table 3.3). Again, there was no difference in

sensitivity to copper for *C. closterium* when cultured in G2 and F2 medium (Table 3.3, Figure 3.1 A).

Table 3.3. Toxicity of nickel and copper to microalgae, using dissolved (0.45 µm filtered) measured concentrations. For each species and metal, data from all definitive tests were combined for statistical analysis. Values in parentheses are 95% confidence limits. All toxicity estimates were calculated using the Weibull model 1.3 or 2.3 (Appendix B, Table B1) in the drc package in R. No observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) values were calculated using Bonferroni's t-test (2-tailed, $p<0.05$) in Toxcalc.

| | **Toxicity estimates (µg/L) (95% confidence limits)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Algal species** | **Nickel** | | | | **Copper** | | | |
| **Dinophyceae** | **EC10** | **EC50** | **NOEC** | **LOEC** | **EC10** | **EC50** | **NOEC** | **LOEC** |
| *Symbiodinium* sp Freud.Clade C | NC | >1620 | 310 | 440 | 3.3 (2.8-3.8) | 5.6 (5.4-5.9) | 3 | 4.3 |
| **Bacillariophyceae** | | | | | | | | |
| *Ceratoneis closterium (G2)* | 3250 (2830-3660) | 6590 (6290-6890) | 3970 | 4070 | 0.97 (0.83-1.1) | 3.3 (3.1-3.6) | 0.8 | 1.4 |
| *Ceratoneis closterium (F2)* | 2539 (2040-3040) | 6090 (5670-6500) | 1610 | 2450 | 0.87 (0.41-1.3) | 2.7 (2.0-3.4) | 0.6 | 2.1 |
| **Coccolithophyceae** | | | | | | | | |
| *Tisochrysis lutea* | 330 (250-410) | 1930 (1780-2090) | 250 | 380 | 2.2 (1.8-2.7) | 5.8 (5.4-6.2) | 1.5 | 2.2 |

NC, not calculated

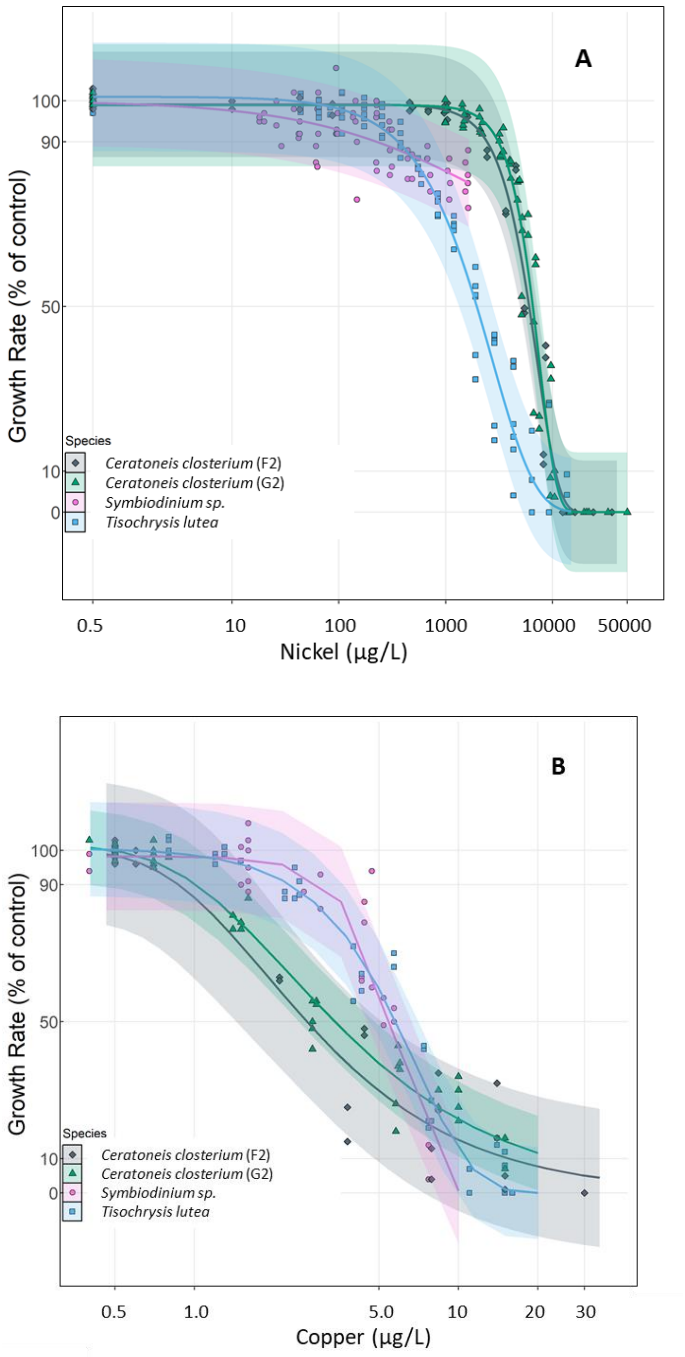



Figure 3.1. Concentration response curves for nickel (A) and copper (B) exposure of tropical marine microalgae, using dissolved (0.45 µm filtered) measured concentrations of metals. Each data point represents 1 individual replicate combined from 3 individual toxicity tests, except for *C. closterium* (F2) where data from 1 rangefinder and 1 definitive test were pooled. The black line indicates the Weibull 1.3 (except for Figure B, Cu, for *C. closterium* (F2 and G2), where Weibull 2.3 model was used) fitted to the data to calculate toxicity estimates. The grey ribbon indicates the 95% prediction interval of the model. Control concentration was set to 0.5 µg/L, which is approximately half the limit of detection for nickel and copper by ICP-AES. Note different scales on x-axis.

## 3.4. Discussion

Prior to this research there were limited nickel toxicity data available for tropical marine microalgae. Initial investigations (presented in Chapter 2 and in Gissi et al. (2016)) found that microalgae were relatively insensitive to nickel compared to other organisms. Effects of nickel toxicity on tropical marine microalgae are typically between 300 – 9500 µg Ni/L, whereas effects on invertebrates have been observed at concentrations as low as 5 µg Ni/L for the tropical marine copepod, *Acartia sinjiensis* (Chapter 2; Gissi et al., 2018). However, the toxicity data for two species of microalgae *Nitzschia closerium* (now known as *Ceratoneis closterium*) and *Isochrysis* sp. (now known as *Tisochrysis lutea*) presented in Chapter 2, were based on two individual toxicity tests only, so it was decided to complete this dataset with a third definitive test with a wider concentration range to capture the full concentration-response curve and to enable comparison with the coral symbiont, *Symbiodinium* sp.

### 3.4.1. Toxicity of nickel to tropical marine microalgae

Our results are similar to those found by Alquezar and Anastasi (2013) for growth rate inhibition by nickel in a cyanobacteria (EC50 of 2300 µg Ni/L). The nickel toxicity data for *C. closterium* and *T. lutea* (Table 3.3) were not significantly different to the original data presented in Chapter 2, except for *C. closterium* (grown in G2 medium). Additional testing has improved the confidence in the EC10 – now 3250 (2830-3660) compared to 6100 (3600-9300) µg Ni/L. There was no difference in the sensitivity of *C. closterium* to nickel when grown in different media types (G2 vs F2, Table 3.3 and Figure 3.1A). Interestingly, the temperate strain of *C. closterium* was more sensitive to nickel than the tropical strain (EC50, 250 µg Ni/L, Florence et al., 1994). However, it is possible that these differences are due to the different initial cell densities used, 1-3 $x10^3$ cells/mL in this study, versus 3-5 $x10^4$ cells/mL used in the study by Florence et al., (1994). Other factors that may influence varying sensitivities across different microalgal species include cell size and shape;

however, in the case of aluminium, this was found to not have a significant impact with both the most and least sensitive species being larger marine diatoms (Gillmore et al., 2016).

The response of *Symbiodinium* to nickel was variable between 100 and 2000 µg Ni/ L and accurate EC10 estimates could not be calculated (Figure 3.1A, Table 4.3). Based on the NOEC values of 310 µg Ni/L and 250 µg Ni/L for *Symbiodinium* and *T. lutea* respectively, these two species appear to have similar sensitivities to nickel while *C. closterium* was less sensitive with NOEC values > 1500 µg Ni/L (Table 4.3.). In toxicity tests with *T. lutea* and *C. closterium*, the nominal test concentration range reached 15 mg Ni/L and 50 mg Ni/L, respectively; whereas the highest concentration tested for *Symbiodinium* was ~2mg Ni/L. These concentrations are well above environmentally relevant concentrations. Typically concentrations of nickel in surface waters are <5 µg Ni/L, although can be up to 2 mg Ni/L in highly polluted areas (Apte et al., 2006; Pyle and Couture, 2012). The solubility limit of nickel in seawater is approximately 70 mg Ni/L (Angel and Apte, CSIRO pers comm). Test concentrations well above environmentally relevant concentrations were used so that reliable toxicity estimates could be calculated. Because the response of *Symbiodinium* was so variable at concentrations <2 mg Ni/L, higher concentrations were not tested for this alga. However, from these data, it is inferred that impacts of nickel on *Symbiodinium* are unlikely to occur in the field.

### 3.4.2. Toxicity of copper to tropical marine microalgae

Copper was more toxic to tropical marine microalgae than nickel, with 10% inhibition in growth rate (EC10) ranging between 0.87 and 3.3 µg Cu/L (Table 3.3.). *Ceratoneis closterium* was the most sensitive species, followed by *T. lutea* then *Symbiodinium*. These species were more sensitive to copper than most other temperate and tropical microalgae (Table 3.4). From the literature, EC50 values for copper range from 0.6 to 88 µg Cu/L. The most (*Minutocellus polymorphus)* and least (*Chaetoceros* sp.) sensitive species are both centric diatoms (Debelius et al., 2009; Levy et al., 2007). The pennate diatom tested in this study *C. closterium* was more sensitive than *Chaetoceros* sp*.* Johnson et al. (2007) tested

the same tropical strain of *C. closterium* grown in G medium, however they reported an EC50 of 40 µg Cu/L, much higher than our study. The most likely cause in the different sensitivities observed for the same strain of microalgae is due to the different cell densities used. Johnson et al. (2007) used a ten-fold higher initial algal cell density and it has been shown that higher cell densities generally result in a lower sensitivity to copper as exudates produced by the microalgae reduce the bioavailability and overall toxicity of copper (Franklin et al., 2002).

Toxicity data for a temperate marine dinoflagellate *Heterocapsa niei,* showed that this species had similar sensitivity to copper as the dinoflagellate, *Symbiodinium*, tested in this study; EC50 values for these species were 4.8 µg and 5.6 µg Cu/L, respectively (Levy et al., 2007, Table 3.4). Interestingly Levy et al. (2007) found that the temperate strain of *C. closterium* was less sensitive to copper than the tropical strains tested in this study; the EC50 value was 18 µg Cu/L, 6-9 times higher than the EC50 value calculated in this study. Similarly, a temperate strain of *Isochrysis* (*Isochrysis galbana,* Debelius et al., 2009) was less sensitive than a tropical isolate (*T. lutea*), tested in this study and by Levy et al. (2007) (Table 3.4). This, together with the different response observed for temperate and tropical *C. closterium* exposed to nickel (Florence et al., 1994, Table 3.4) highlights the importance of using regionally-relevant species when acquiring toxicity data for WQG development.

In all toxicity tests there was a significant loss of dissolved copper in test solutions (between 3-100%). The most significant decrease in dissolved copper occurred at the lowest nominal concentrations (94-100% loss at 0.5 and 1 µg Cu/L). These concentrations are close to the limit of detection (0.1 µg Cu/L) and background concentration of copper in seawater (<0.72 µg Cu/L); this makes accurate measurement at these levels challenging. Low cell densities were used in all bioassays to minimise copper depletion in solution by algal exudates (Franklin et al., 2002); however copper loss can still occur due to absorption to glass flasks and adsorption or absorption by algal cells (Levy et al., 2007).

### 3.4.3. Toxicity testing with *Symbiodinium* sp.

Data on the effect of metals to *Symbiodinium* sp. is scarce. This genus of dinoflagellate plays a crucial role in tropical marine systems. *Symbiodinium* form a unique and vital relationship with corals, supplying them with >95% of their energy (Boulotte et al., 2016). *Symbiodinium* are dinoflagellates and free-swimming phytoplankton found in the water column, prior to establishing a symbiotic relationship with corals. During their free swimming stage, *Symbiodinium* could potentially be exposed to and impacted by anthropogenic contaminants or stressors (Rodriguez et al., 2016). Additionally, it has been found that *Symbiodinium* accumulates most metals in greater concentrations than coral tissue (Reichelt-Brushett and McOrist, 2003). If coral-algal symbioses become compromised by stressors such as increased temperature, or changes in water quality (e.g. metal contamination), corals expel the algal symbionts or “bleach” and thus lose a major energy source (Bielmyer et al., 2010; Reichelt-Brushett and McOrist, 2003).

To our knowledge, there are no studies which have investigated the effects of nickel toxicity to *Symbiodinium*. Kuzminov et al. ( 2013) demonstrated that copper, zinc, cadmium and lead disrupted photosynthesis in *Symbiodinium*. This can reduce growth and the supply of energy to corals. In an earlier study by Goh and Chou (1997), 40 µg Cu/L significantly reduced the growth of *Symbiodinium*. These results are significantly different to the results presented in this study where the growth rate of *Symbiodinium* was inhibited by 10% following exposure to 3.3 µg Cu/L. However, it is difficult to make comparisons because Goh and Chou did not indicate the strain of *Symbiodinium* used in the study nor did they calculate toxicity estimates. In addition, their tests were conducted in a nutrient-rich medium which is known to reduce the toxicity of metals through chelation and adsorption. Although in our study the *Symbiodinium* was not washed to remove culture medium (as was done for other microalgal species), the volume of algal inoculum added to the test flask was <0.5% of the total test volume. It is therefore unlikely that the small culture medium carryover reduced the

bioavailability of nickel and copper in the test solutions to the same extent as conducting the tests in full culture medium.

Due to the high ecological importance of *Symbiodinium* in tropical marine ecosystems, understanding the impacts of metal contaminants on this taxon is highly relevant. This is the first report on the toxicity of nickel to *Symbiodinium* and these data will be valuable in contributing to the development of an ecologically-relevant WQG for nickel.

Table 3.4. Toxicity of nickel and copper to temperate and tropical marine microalgae (adapted from Gissi et al., 2015)

| | | Nickel | | Copper | | | |
|---|---|---|---|---|---|---|---|
| | | µg/L | | | | | |
| **Class** | **Algal Species** | **EC50[a]** | **NOEC[b]** | **EC50[a]** | **NOEC[b]** | **Climatic Region** | **Reference** |
| Cyanobacteriaceae (cyanobacteria) | *Cyanobium* sp. | 2300 | | | | Tropical | Alquezar and Anastasi (2013) [c,f] |
| Bacillariophyceae (diatoms) | *Nitzschia closterium*[h] | | | 18 (6-30) | 4.4 | Temperate | Levy et al. (2007) [c, f] |
| | | 250 | NR | | | Temperate | Florence et al. (1994) [d,f] |
| | | >500 | NR | | | Tropical | |
| | | | | 40 ± 4[d,e] | NR | Tropical | Johnson et al. (2007) [d,e] |
| | | 6590 | 3970 | 3.3 (3.1-3.6) | 0.8 | | |
| | *Ceratoneis closterium* | (6290-6890) | | | | Tropical | This study [c,e] |
| | | 6090 | 1610 | 2.7 (2.0-3.4) | 0.6 | | |
| | *Ceratoneis closterium* | (5670-6500) | | | | Tropical | This study [c,f] |
| | *Phaeodactylum tricornutum* | | | 8 (5-8.3) | <1.5 | Temperate | Levy et al. (2007) [c,f] |
| | *Minutocellus polymorphus* | | | 0.6 (0.5-0.8) | <0.2 | Temperate | Levy et al. (2007) [c,f] |
| | *Chaetoceros* sp. | | | 88 ± 10 | NR | Temperate | Debelius et al. (2009) [d] |
| Dinophyceae (Dinoflagellate) | *Heterocapsa niei* | | | 4.8 (3.5-7.2) | NR | Temperate | Levy et al. (2007) [c, e] |
| | *Symbiodinium* sp. Freud.Clade C | NC | 310 | 5.6 (5.4-5.9) | 3 | Tropical | This study [c,g] |
| Coccolithophyceae | *Isochrysis* sp.[i] | | | 4 (3.8-4.2) | <1.1 | Tropical | Levy et al. (2007) [c,f] |
| | *Isochrysis galbana* | | | 58 ± 30 | NR | Temperate | Debelius et al. (2009) [d] |
| | | 1930 | 250 | 5.8 (5.4-6.2) | 1.5 | | |
| | *Tisochrysis lutea* | (1780-2090) | | | | Tropical | This study [c,f] |

[a] EC50, Effect Concentration, concentration of metal to cause a 50% inhibition in population growth rate, compared to controls. Values in parentheses are 95% confidence limits or ± 1 standard deviation

[b] No observable effect concentration, test concentration which is not statistically different to control

[c] Cell density = $10^3$ cells/mL

[d] Cell density = $10^4$ cells/mL

[e] Study used alga that was cultured in G medium (Loeblich and Smith, 1968)

[f] Study used alga culture in F medium (Gulliard et al., 1962)

[g] Study used alga culture in F medium (Gulliard et al., 1962), with 2 x Fe concentration

[h] Now known as *Ceratoneis closterium*

[i] Now known as *Tisochrysis lutea;* NC, not calculated

NR not reported

## 3.5. Conclusion

Microalgae play a crucial role in tropical marine ecosystems; as a food or energy source for higher trophic organisms, and as sensitive indicators of environmental change. This study investigated the toxicity of nickel and copper to three different species of microalgae. Overall, copper was more toxic than nickel, with growth rates inhibited by 10% at 0.87-3.3 µg Cu/L. *Ceratoneis closterium* was more sensitive to copper than *T. lutea* and *Symbiodinium*. Based on NOEC values, *T. lutea* was more sensitive to nickel followed by *Symbiodinium* and *C. closterium.* Notably, this is the first report on the toxicity of nickel to a coral endosymbiont, *Symbiodium*. Future work to contribute to a mechanistic understanding of nickel toxicity to microalgae could include gene expression studies.

Inclusion of toxicity data for microalgae in WQG development is necessary, given their key role in aquatic systems. These high-quality toxicity data for three different tropical species of microalgae will contribute to the development of a WQG for nickel in tropical marine waters. Other key taxa to include in tropical marine WQG development include crustaceans, molluscs and cnidarians. These are addressed in the following chapters.

# 4. ASSESSING THE CHRONIC TOXICITY OF NICKEL TO A GASTROPOD AND TWO CRUSTACEANS

## Context statement

Chapter 3 provided nickel toxicity data for relevant primary producers (microalgae), however, data are also required for higher trophic organisms. The gap analysis presented in Chapter 2 identified molluscs (including gastropods) and crustaceans as key data gaps in the availability of chronic nickel toxicity data for tropical marine species. The toxicity of nickel to three tropical marine invertebrates, the gastropod *Nassarius dorsatus*, barnacle *Amphibalanus amphitrite* and copepod *Acartia sinjiensis* was investigated. All toxicity tests used chronic endpoints, namely larval growth, metamorphosis (transition from nauplii to cyprid larvae) and larval development for the snail, barnacle and copepod respectively. Copper was also tested for quality assurance purposes and to allow for comparisons with previous studies. The work presented in this chapter has been adapted from the below cited publication[3].

[3]Gissi, F., Stauber, J.L., Binet, M.T., Trenfield, M.A., Van Dam, J.W., Jolley, D.F. (2018). Assessing the chronic toxicity of nickel to a tropical marine gastropod and two crustaceans. Ecotoxicology and Environmental Safety, 159, 284-292. https://doi.org/10.1016/j.ecoenv.2018.05.010. I conducted all bioassays, with assistance from M.T. Binet (for copepods), M.A. Trenfield (for snails) and J.W. Van Dam (for barnacles). I completed the analysis of metal samples and statistical analysis, analysed data and prepared the manuscript for publication.

## 4.1. Introduction

The review in Chapter 2 compiled and quality-checked available nickel toxicity data for tropical marine species. Only six high quality data, representing four taxonomic groups were found and this was insufficient to derive a water quality guideline value for tropical systems. Key data gaps identified in the review included cnidarians, molluscs, crustaceans, echinoderms, macroalgae and fish. Chapter 3 built on existing data for microalgae, for which preliminary data were presented in Chapter 2. The aim of this chapter is to further contribute to the body of chronic nickel toxicity data for tropical marine species, specifically for molluscs (gastropods) and crustaceans. These taxonomic groups have high ecological importance in tropical regions because of the role they play in the food web, and contributors to species richness and biodiversity which is highest in the tropics (Bouchet et al., 2002; Humes, 1994; Roberts et al., 2002). Additionally crustaceans and molluscs are among the most sensitive species to nickel exposure in temperate systems (Bielmyer et al., 2006; Deforest and Schlekat, 2012; Niyogi et al., 2014).

The effects of nickel on a gastropod, the channelled dog whelk *Nassarius dorsatus* and two crustaceans, the purple-acorn barnacle *Amphibalanus amphitrite* and the copepod *Acartia sinjiensis* were investigated. All three species tested in this study are found in tropical marine environments of the SEAM region. *Nassarius dorsatus* is most common in tropical North Australia, and has also been reported in coastal waters off Malaysia, Indonesia, Papua New Guinea, Fiji and the Philippines (Trenfield et al., 2016). *Amphibalanus amphitrite* is a common biofouling organism, widely distributed across tropical to temperate waters, in the mid to low intertidal zone (Desai et al., 2006; van Dam et al., 2016). This species has been widely used as a model species in biofouling and ecotoxicity tests (Rittschof et al., 1992; van Dam et al., 2016). *Acartia sinjiensis* is found in tropical and sub-tropical brackish to marine waters in Australia and some other locations within the Asia-Pacific (Gissi et al., 2013). This

species of copepod is a common food source for many higher trophic organisms (Camus and Zeng, 2008).

The objectives of this study were to assess larval growth rate for the snail, metamorphosis (successful transition from nauplii to cyprid larvae) for the barnacle and larval development for the copepod, to determine the chronic toxicity of nickel and copper to each of the three species. The definition of chronic toxicity is based on the Australian and New Zealand Water Quality Guidelines which states that chronic toxicity is "*an adverse sub-lethal effect on a sensitive early life stage"* (Batley et al., 2018, Chapter 1, section 1.4.2.). The toxicity data presented here will contribute to the further development of reliable WQGV for nickel in tropical marine waters.

## 4.2. Methods

### 4.2.1. General laboratory techniques and reagents

Snail and barnacle toxicity tests were conducted at the Australian Institute of Marine Science (AIMS, Darwin, Northern Territory, Australia). One range-finding and four definitive tests were conducted each for the snail and barnacle. Three definitive copepod tests were carried out at the CSIRO Land and Water laboratories in Sydney, NSW, Australia.

All glassware and plastic containers used in the tests were washed and prepared as discussed in Chapter 3 (Section 3.2.1). Glass funnels used in barnacle tests were silanized (Coatasil, Ajax, Finechem), approximately 2 weeks prior to testing and thoroughly rinsed with demineralised water, soaked in 10% nitric acid for 24 h, and finally rinsed again with demineralised water, followed by high purity water. Metal stocks of 5 and 100 mg Cu/L and 10 and 100 mg Ni/L were prepared as per Chapter 3 (Section 3.2.1).

For the snail and barnacle tests, water quality parameters including dissolved oxygen (DO), pH, conductivity and salinity were measured using a multi-probe (Hach multiprobe HQ40d), which was calibrated each day according to the manufacturer's instructions. For snail tests, measurements were taken on Day 0 and every 24 h. For barnacle tests, measurements

were taken on Days 0 and 4. In all copepod tests physico-chemical measurements were taken as detailed in Chapter 3 (Section 3.2.1). Water quality parameters were measured on Day 0, Day 2 before and after renewal and on Day 3. Temperature was recorded in all toxicity tests throughout the exposure.

### 4.2.2. Toxicity tests with the snail *Nassarius dorsatus*

Culturing, larval hatching and toxicity testing with *N. dorsatus* followed the methods described in Trenfield et al. (2016), and utilised the same broodstock of snails. In brief, egg batches were laid on polystyrene tubes positioned in the broodstock tank. Prior to the eggs hatching, tubes were transferred to a clean container with filtered seawater. The egg batches were maintained at 29°C and after ~5 days larvae hatched, then were fed once per day with a mixture of the microalgae *Chaetoceros muelleri* and *Rhodomonas salina* for 2 days.

Seawater for snail tests was collected in 20 L polyethylene containers from Nightcliff Jetty, Northern Territory, Australia (12°22’59”S, 130°50’56”E), at high tide and filtered (0.45-µm filter, Quickfilter groundwater cartridge; Thermofisher Scientific) immediately upon return to the laboratory. Filtered seawater was stored at 4°C in the dark.

Treatment solutions were prepared using seawater in 2 L HDPE bottles 24 h prior to test commencement. Across four individual toxicity tests, nominal nickel concentrations were in the range of 50 – 1500 µg Ni/L. In the single copper test, nominal concentrations were 2, 4, 8, 12 and 20 µg Cu/L. Physico-chemical parameters of treatment solutions were measured each day, and on day 4, parameters were measured in one replicate test container for each treatment.

On Day 0, unwashed axenic cultures of the microalgae *C. muelleri* and *R. salina* were added to each test container to give a final concentration of 1 x$10^4$ cells/mL of each species, then 100 mL of each treatment solution was dispensed into the relevant test container, and 10 larvae (2-day old) were gently added using a wide-mouthed glass pipette. Test containers were placed in a temperature-controlled cabinet set to 28°C (as per Trenfield et al., 2016).

The volume of microalgae added to the test containers each day was <4% of the total test volume. Five to six metal treatments were tested alongside a control, with three replicates per treatment. New treatment solutions were made every 24 h and prior to the addition of new food and solutions, each test container was observed, and the number of living larvae were counted and checked under a stereomicroscope. Larvae without a heartbeat were classified as dead and were removed from the test containers. Sub-samples were taken from the bulk treatment solutions on Day 0, Day 2 and from one replicate container of each treatment on Day 4 to measure total and dissolved metals (see Chapter 3, Section 3.2.4,). Throughout the exposure, test vessels were randomized twice a day.

On Day 0, 20 larvae from the batch of larvae used in the test were selected and photographed to determine the average starting length as described by Trenfield et al. (2016). Images were captured using a Leica DFC320 camera attached to a Leica DM4000B microscope at a magnification of 250×. On Day 4, after observing larval survival, five larvae from each test container were selected at random and photographed for length measurements. Growth rate (µm/d) was calculated as (Day 4 length – Day 0 length)/4. These values were converted to percent of control for statistical analysis.

### 4.2.3. Toxicity tests with the barnacle *Amphibalanus amphitrite*

Culturing, broodstock spawning, and toxicity testing methods followed those established in van Dam et al. (2016) and utilised the same broodstock.

Seawater from the aquaria in the AIMS laboratory, was UV-sterilized and filtered through a 0.5 µm polypropylene cartridge filter, for use in the barnacle tests. Treatment solutions were prepared using seawater in 1 L HDPE bottles 24 h before test commencement to ensure equilibration. Treatment solutions were made 1.5 times more concentrated than nominal values to allow for further dilution upon addition of nauplii in seawater to the test containers. Across four individual toxicity tests, five nominal concentrations of nickel were tested ranging from 50 – 500 µg Ni/L. In the single copper test, the nominal concentrations tested were 20, 40, 60, 80 and 100 µg Cu/L. Sub-samples were taken from the bulk solutions for total and

dissolved metals (see Chapter 3, Section 3.2.4,). In each toxicity test, five metal treatments were tested alongside a control, with four replicates per treatment. Physico-chemical parameters were measured in the bulk treatment solutions on Day 0, and from one replicate of each treatment on Day 4.

On Day 0, the microalga *C. muelleri* was filtered through a 48 µm mesh, centrifuged and washed with seawater to remove culture media. Cell densities were determined using a haemocytometer, and 1 x$10^5$ cells/mL were added to each test container. This process was followed each day using the same culture stock of algae to provide food to the nauplii throughout the exposure. The volume of algae added to the test containers each day was low (<0.5%).

Glass funnels were set up on aeration lines and 100 mL of the treatment solutions were added to the relevant funnels, as well as $10^7$ cells of concentrated *C. muelleri*. Funnels were gently aerated throughout the exposure. Following induction of spawning, nauplii ≤2-h old were collected from the broodstock tank. One polystyrene jar, containing 40 mL of seawater and 5 x $10^6$ cells of *C. muelleri* was prepared for each funnel. Nauplii were transferred to a Petri dish and using a stereo microscope, 75 nauplii were gently pipetted from the Petri dish into each polystyrene jar. One jar was then transferred into each funnel and the jar was rinsed with 10 mL of seawater that was also added to the funnel to ensure all nauplii were transferred. Funnels were capped with plastic Petri dish bases. Funnels were kept in the aquaria at 29°C, under the same conditions described in van Dam et al. (2016). Randomising test vessels during the exposure was not possible (van Dam et al. 2016).

At 96 h, funnels were disconnected from the aeration line and the contents drained onto a 150 µm nitrile mesh. Using a stereomicroscope, the number of cyprid larvae (the larval stage prior to settlement) was recorded and the success of metamorphosis was calculated as the number of cyprid larvae at 96 h/ number of nauplii at 0 h. These values were converted to percent of control for statistical analysis. One sample was taken from one replicate of each

treatment to measure physico-chemical parameters and to sub-sample for total and dissolved metals (see Chapter 3, Section 3.2.4,).

### 4.2.4. Toxicity tests with the copepod *Acartia sinjiensis*

Copepods were cultured according to Gissi et al. (2013). Toxicity test methods followed the protocol established by (Binet et al., 2019). Mass cultures of *A. sinjiensis* were obtained from Queensland Department of Primary Industries Northern Fisheries Centre, originally isolated from plankton collections offshore from Townsville, Queensland (Gissi et al., 2013). Seawater used in tests was collected from Cronulla, NSW, Australia, filtered and stored as described in Chapter 3 (Section 3.2.1).

Copepod larval development tests were semi-static with a partial renewal on Day 2. Physico-chemical parameters were recorded, and sub-samples of each treatment were collected for total and dissolved metal analyses (see Chapter 3, Section 3.2.4) at test commencement (0 h), before and after renewal (48 h), and again at test completion. Three individual nickel toxicity tests were performed encompassing the nominal concentration range of 4 – 16 µg Ni/L. For the three individual copper tests, nominal concentrations ranged from 1 – 12 µg Cu/L. In each toxicity test five metal treatments were tested alongside a control with four replicates per treatment. The control treatment had eight replicates to allow for additional replicates to be sacrificed when checking the larval development ratio (LDR) on Day 3.

Bulk solutions of nickel and copper treatments were prepared in clean acid-washed polycarbonate containers in filtered seawater with added microalgal food on Day 0 ($8 \times 10^4$ cells/mL *Tisochrysis lutea*/mL and $0.63 \times 10^4$ cells/mL *Tetraselmis chuii*) and Day 2 ($4 \times 10^4$ cells/mL *Tisochrysis lutea*/mL and $0.31 \times 10^4$ cells/mL *Tetraselmis chuii*). Microalgae were centrifuged prior to addition to treatments to remove algal growth medium. The total volume of algae added to each test container was <0.5%. On Day 0, 60 mL of each test treatment was dispensed into clean acid-washed 250 mL polycarbonate containers and allowed to equilibrate to 30°C for 1 h in the test cabinet.

Adult copepods were isolated 24 h prior to test commencement. The isolated adults were fed a mixture of microalgae, *T. lutea* and *Proteomonas sulcata* (1:8). On the day of test commencement (Day 0), eggs were isolated from adult cultures, rinsed with seawater into a 100 mL beaker and returned to the culture cabinet until required. Prior to inoculation of eggs, any nauplii that hatched were removed by rinsing with high purity water and the eggs concentrated again in seawater (Binet et al., 2019). This egg concentrate was counted on the microscope (Olympus SZX10, Japan, 10x magnification) to determine the egg density and volume of inoculum such that approximately 40-60 eggs were added to each test container with 60 mL of treatment solution. The inoculated test was placed in the test cabinet and the time recorded. Throughout the exposure, test containers were randomized in the cabinet twice a day.

On Day 2, a partial renewal was completed by replenishing each test container with 120 mL of freshly prepared test treatment solution with algae. The test was terminated after approximately 80 h, or when a minimum of 50% of animals in the controls had developed into copepodites (LDR ≥ 50%) (ISO, 2015; OECD, 2007). Following measurement of physico-chemical parameters and sub-sampling for metals, all containers were fixed with formalin and stained with Rose Bengal (Binet et al., 2019). These containers were stored in the dark at 4°C for a minimum of 24 h before LDR assessments were performed.

Fixed samples were filtered through a 54 µm sieve, rinsed with high purity water and counted under a dissecting microscope (Olympus SZX10, Japan, x10 magnification) to determine number of eggs, nauplii and copepods. The larval development ratio was calculated (LDR = ∑copepodite/∑(copepodite + nauplii)). These values were converted to percent of control for statistical analysis.

The samples were also analysed using a zooplankton analyser (Zooscan 3, Hydroptic Model #ZSCA03, France). The scanned data were processed and analysed using Zooprocess 7.22 (http://www.zooscan.obs-vlfr.fr/rubrique.php3?id_rubrique=49?lang=en) and Plankton

Identifier v1.3.4 (http://www.obs-vlfr.fr/~gaspari/Plankton_Identifier/index.php) as described by Binet et al. (2019).

Use of the Zooscan to count and identify the larval stage of each animal detected by the software as either eggs, naupliar stage 1 and 2 (N1N2), naupliar stage 3 and 4 (N3N4), naupliar stage 5 and 6 (N5N6), copepodite stages 1, 2 and 3 (C1C2C3) and copepodite stages 4, 5 and 6 (C4C5C6) has been validated in our laboratories (Binet et al., 2019). These groupings were required to achieve the lowest error rate in the software's ability to accurately categorise animals. In this study, toxicity estimates were based on the larval development ratio determined by microscope counts. The Zooscan data were used to investigate the number of animals at each stage of development.

#### 4.2.5. Chemical and statistical analysis

Analysis of total and dissolved metals was performed as described in Chapter 3 (Section 3.2.4). For snail and copepod tests, the time weighted average (TWA) concentrations were used in statistical analyses. For barnacle tests the mean dissolved concentration (from Day 0 and 4) was used in statistical analysis, because only two time points were sampled. For each species, data from all four tests (or three tests for the copepod) were combined and analysed as discussed in Chapter 3 (Section 3.2.5). The models for statistical analysis were selected based on the AIC value (Appendix C, Table C1). Dissolved organic carbon (DOC) in seawater used in tests was sampled and analysed as described in Chapter 3 (Section 3.2.4).

### 4.3. Results

#### 4.3.1. Quality control

The background concentrations of metals in the seawater used in all tests were generally below the limit of detection (LOD) (Appendix C Table C2-4). Metals in seawater used for culturing were also below LOD (data not shown). For all three species, in all toxicity tests, physico-chemical parameters remained within the expected ranges. For the snail, pH ranged

from 8.1 - 8.4, salinity ranged from 35-37‰; for the barnacle, pH ranged from 8.2-8.4, salinity was 33-34‰; and for the copepod, pH ranged from 8.1-8.2 and salinity ranged from 34-36‰. In all tests, DO was maintained above 90%. The temperature was maintained within 0.5°C of the required temperature; 28°C for the snail, 29°C for the barnacle and 30°C for the copepod. The average concentration of DOC in the filtered seawater used in the snail tests was 0.7 ± 0.1 (SD) mg/L, while in barnacle and copepod tests it was 0.8 ± 0.2 mg/L. This is within the range found for tropical coastal waters, with concentrations of DOC in SEAM waters around 0.5 – 4 mg/L (Ross Smith, pers. comm.).

In all tests, the measured dissolved nickel concentrations were within 12% of the nominal values, and in most cases dissolved nickel made up 95-100% of the total fraction. The exception was in the copepod test, where the difference between nominal and measured nickel concentrations (based on a time-weighted average, TWA) was up to 21% and the dissolved nickel was between 88-100% of the total fraction. In all copper tests, up to 67% of dissolved metal was lost during the exposure. Dissolved measured TWA values for copper were within 64% of nominal values. The fraction of dissolved copper in all tests ranged from 58-100% (Appendix C, Table C5-7).

All snail, barnacle and copepod tests in this study met the criteria for control treatments (Trenfield et al., 2016; van Dam et al., 2016; Binet et al. 2019). In this study the mean growth rate of snail larvae in control treatments, across four individual tests was 62 ± 8.4 µm/d (±SD). Across four individual toxicity tests with the barnacle, the mean % of successfully transitioned larvae in control treatments was 86 ± 2.6% (±SD). Across three individual copepod tests the average larval development ratio in the control treatments was 68 ± 10% (±SD).

### 4.3.2. Toxicity of nickel to the snail, barnacle and copepod

Based on EC10, EC20 and EC50 (± 95% CLs) values of 5.5 (5.0 - 6.0) µg Ni/L, 6.6 (6.1-7.0) and 8.6 (8.3 - 8.9) µg Ni/L respectively, the copepod was the most sensitive species to nickel following an 80-h exposure. The snail and barnacle were of similar sensitivity to nickel at low

concentrations, i.e., EC10 values were 64 (37-91) µg Ni/L and 67 (53-80) µg Ni/L, respectively, following a 96-h exposure. However, the EC50 values for these two species were substantially different: 478 (420-536) µg Ni/L and 171 (160-182) µg Ni/L for the snail and barnacle, respectively (Table 4.1). The concentration-response curves for nickel toxicity to snail, barnacle and copepod are shown in Figure 4.1 A-C.

### 4.3.3. Toxicity of copper to the snail, barnacle and copepod

Copper was more toxic than nickel to all species tested (Table 4.1, Figure 4.2). The copepod was the most sensitive species with an EC10 of 1.4 (1.2-1.8) µg Cu/L, followed by the snail (EC10 of 3.7 (2.7-4.7) µg Cu/L) and the barnacle (EC10 of 10 (5-15) µg Cu/L) (Table 4.1). The toxicity estimates in this study are similar to those in previous studies with the snail (Trenfield et al., 2016) and the barnacle (van Dam et al., 2016). Toxicity estimates for the copepod are within the ranges reported in our laboratory database (Binet et al. 2019), showing that these test protocols are robust and reproducible.

Table 4.1. Toxicity of nickel and copper to invertebrates, using dissolved (0.45 µm filtered) measured concentrations. Values in parentheses are 95% confidence limits. All toxicity estimates were calculated using the Weibull model 1.3 in the drc package in R.

| | **Toxicity estimates (µg/L) (95% confidence limits)** | | | | | |
|---|---|---|---|---|---|---|
| | **Nickel** | | | **Copper** | | |
| | **EC10** | **EC20** | **EC50** | **EC10** | **EC20** | **EC50** |
| **Gastropod** | | | | | | |
| *Nassarius dorsatus* | 64 (37-91) | 140 (100-180) | 480 (420-540) | 3.7 (2.7-4.7) | 4.8 (3.9-5.7) | 7.1 (6.4-7.8) |
| **Crustaceans** | | | | | | |
| *Amphibalanus amphitrite* | 67 (53-80) | 97 (84-110) | 170 (160-180) | 10 (5-15) | 15 (9.4-20) | 26 (22-30) |
| *Acartia sinjiensis* | 5.5 (5.0-6.0) | 6.6 (6.1-7.0) | 8.6 (8.3-8.9) | 1.4 (1.2-1.8) | 1.9 (1.6-2.2) | 2.8 (2.6-2.9) |

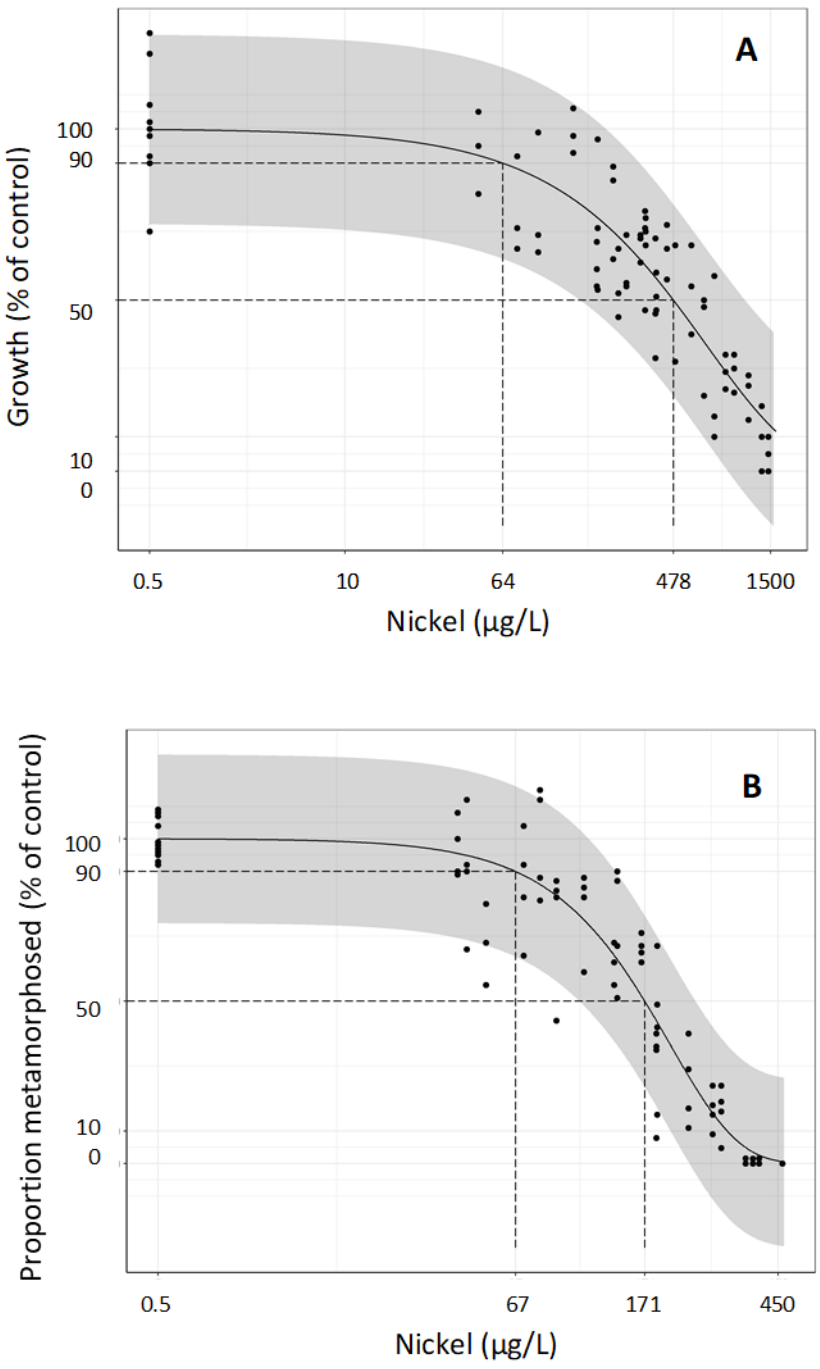



Figure 4.1. Toxicity of nickel to A) the snail *Nassarius dorsatus*, B) the barnacle *Amphibalanus amphitrite* and C) the copepod *Acartia sinjiensis*. For A and B, each point represents 1 replicate from 4 individual toxicity tests; for C, each point represents 1 replicate from 3 individual toxicity tests. The black line indicates the Weibull 1.3 model, fitted to the data to calculate toxicity estimates. The grey ribbon indicates the 95% prediction interval of the model, and the dashed lines point to the 10% and 50% effect concentrations, calculated from the model. Nickel values are dissolved (0.45 µm filtered) measured concentrations; control concentration was set to 0.5 µg Ni/L, which is approximately half the limit of detection for nickel by ICP-AES. Note different scales on x-axis, different toxicity endpoints on y-axis are adopted for different test species.

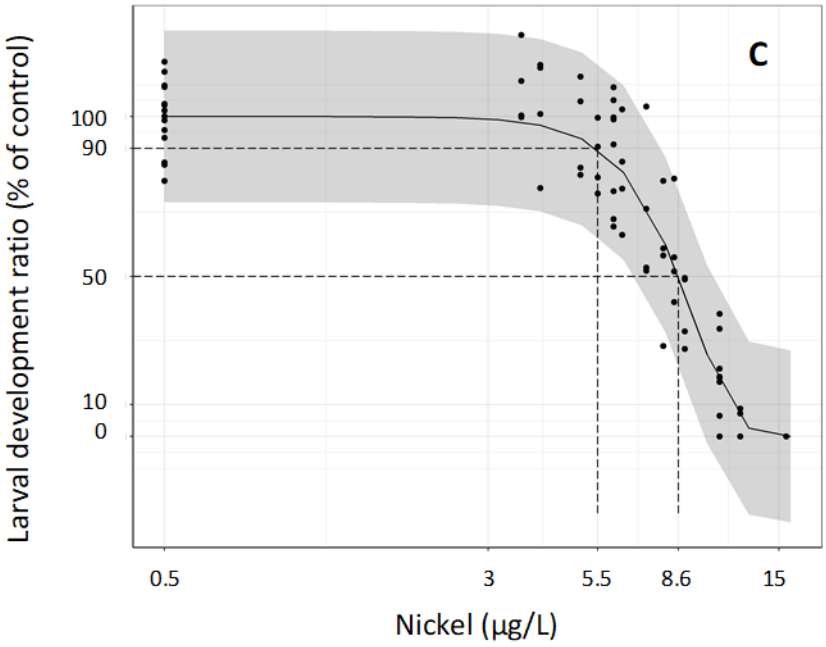
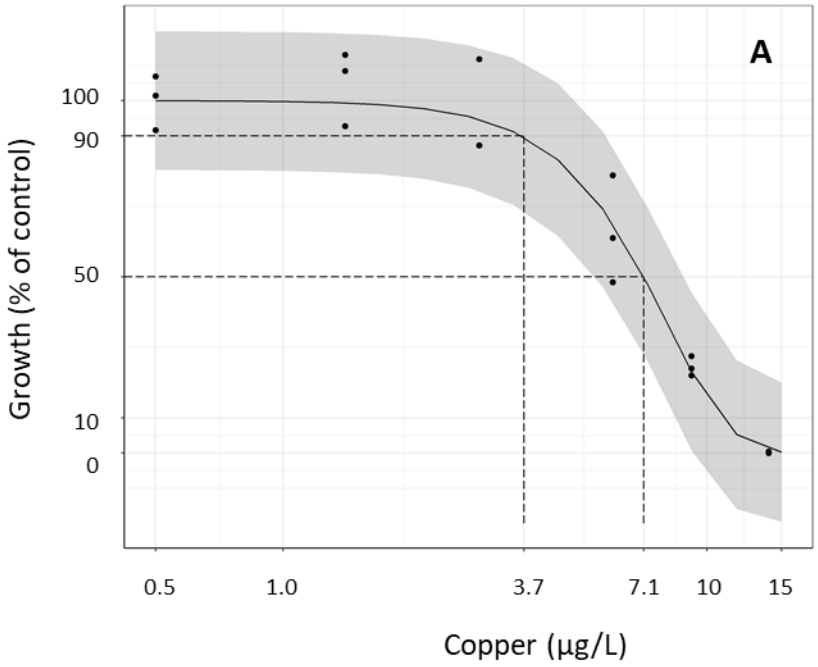



Figure 4.1. Continued.



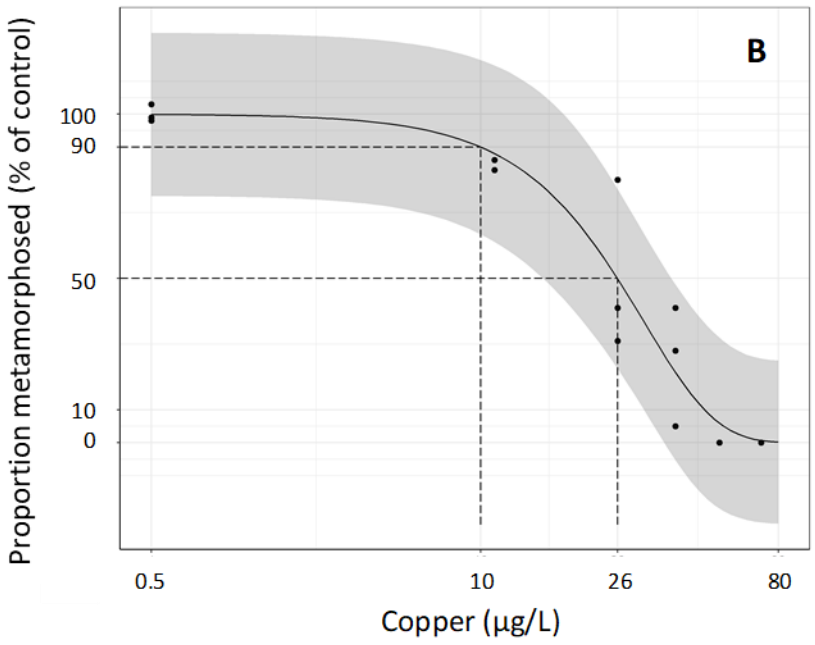




Figure 4.2. Toxicity of copper to A) the snail *Nassarius dorsatus*, B) the barnacle *Amphibalanus amphitrite* and C) the copepod *Acartia sinjiensis*. For A and B, each point represents 1 replicate from 1 individual toxicity tests; for C, each point represents 1 replicate from 3 individual toxicity tests. The black line indicates the Weibull 1.3 model, fitted to the data to calculate toxicity estimates. The grey ribbon indicates the 95% prediction interval of the model, and the dashed lines point to the 10% and 50% effect concentrations, calculated from the model. Copper values are dissolved (0.45 µm filtered) measured concentrations; control concentration was set to 0.5 µg Cu/L, which is approximately half the limit of detection for copper using ICP-AES. For Figure C, the value is set to 0.05 µg Cu/L, approximately half the limit of detection of ICP-MS. Note different scales on x-axis, different toxicity endpoints on y-axis are adopted for different test species.

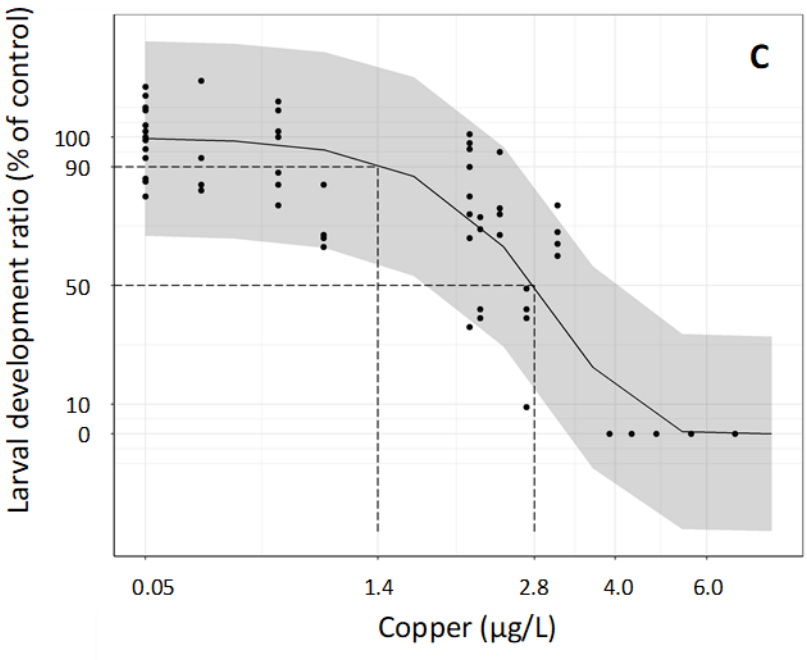



Figure 4.2. Continued.

### 4.3.4. The effect of nickel and copper on the different stages of copepod development

Following 80-h exposure, 58-72% of copepods in control treatments had developed to the first stages of copepodite (C1C2C3), 22-33% were at nauplii stages 5 and 6 (N5N6), 5-7% were at nauplii stages 3 and 4 (N3N4) and 2-3% were at nauplii stages 1 and 2 (N1N2) (Figure 4.3). Nickel inhibited the development of nauplii into copepodites. As the concentration of nickel increased, the proportion of copepods at stages C1C2C3 decreased, and at the highest concentration tested (16 µg Ni/L), there were no copepodites (Figure 4.3) and only 3% of nauplii were at stages N5N6. The proportion of nauplii at N3N4 increased with increasing nickel concentration from 5% at 3.7 µg Ni/L to 86% at 16 µg Ni/L. On average, the proportion of N1N2 nauplii slightly increased with increasing nickel concentration.

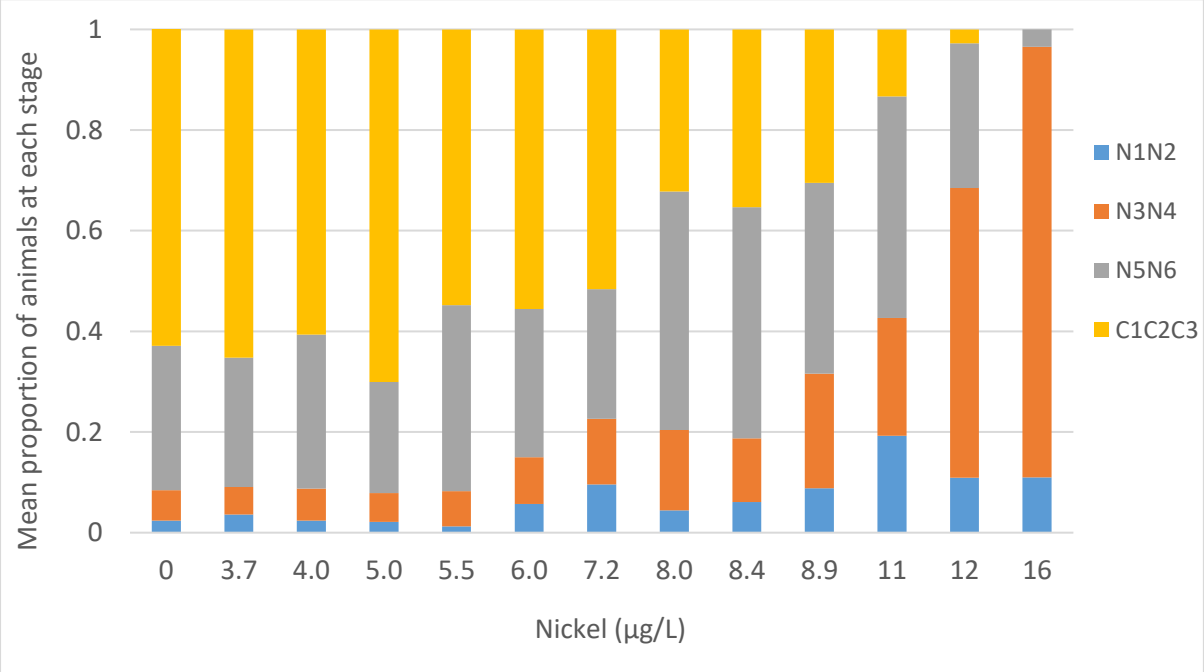



Figure 4.3. The effect of nickel on the different stages of copepod development over ~80 h, expressed as the proportion of animals at each stage of development as determined by the Zooscan. Data were compiled from 3 individual toxicity tests (n= 3 – 9).

## 4.4. Discussion

### 4.4.1. Toxicity of nickel to gastropods

When comparing the EC10 and EC20 values, the snail and barnacle demonstrated similar sensitivity to nickel. However, based on the EC50 values, the snail *Nassarius dorsatus* was the least sensitive to nickel out of the three species tested. Two previous studies have reported acute toxicity for the tropical marine snail *Babylonia areolata*. Survival of adult snails after 96-h exposure was inhibited by 50% (LC50, 95% confidence limits) at 36000 (35000-28000) µg Ni/L and for juvenile snails at 200 (110-340) µg Ni/L, respectively (Hajimad and Vedamanikam, 2013; Vedamanikam and Hayimad, 2013). No chronic nickel toxicity data were available for marine gastropods (temperate or tropical), but, for temperate marine bivalves, EC10 values ranged from 61 – 431 µg Ni/L (Deforest and Schlekat, 2012). *Nassarius dorsatus* is therefore the most sensitive marine gastropod to nickel so far reported.

### 4.4.2. Toxicity of nickel to crustaceans

This is the first report on the toxicity of nickel to a tropical marine barnacle. Previous acute studies reported EC50 values for nickel to tropical marine crustaceans ranging from 7.2 – 18000 µg Ni/L (Gissi et al., 2016, Chapter 2), yet chronic toxicity data were far more limited. One study showed that after an 11-d exposure, growth in a tropical marine shrimp, *Artemia franciscana* was reduced (by approximately 50%) at 3 µg Ni/L, but toxicity estimates were not reported (Asadpour et al., 2013). Chronic nickel toxicity data were available for several temperate marine and estuarine crustaceans. Reproductive output in three species of euryhaline copepods was reduced at 10 µg Ni/L (Mohammed et al., 2010). Reproduction in the temperate copepod *Acartia tonsa* was reduced by 20% at 2.4 µg Ni/L after a 7-d dietary exposure to nickel (Bielmyer et al., 2006). Survival of *A. tonsa* nauplii after 7-d exposure to nickel was reduced by 50% at 39 µg Ni/L, and egg hatching success after 4-d exposure to 25 – 100 µg Ni/L was reduced by 14 - 20% (Zhou et al., 2016). Gorbi et al. (2012) found similar results for the same species showing that nauplii survival was reduced by 50%

between 29 – 76 µg Ni/L after 7-d exposure. The temperate marine mysid shrimp, *Mysidopsis intii*, demonstrated similar sensitivity to nickel; 28-d growth was reduced by 10% at 45 µg Ni/L (Deforest and Schlekat, 2012). Based on the current chronic toxicity data available for nickel, crustaceans, compared to all other marine organisms, showed the highest sensitivity to nickel.

In this study, the individual nauplii and copepodite stages of *A. sinjiensis* were assessed and showed that concentrations ≥8 µg Ni/L reduced the number of copepodites. This important developmental stage (from nauplii to copepodite) requires extensive physiological change and is often the most sensitive developmental window for *Acartia* species (OECD, 2007). This test is terminated at approximately 80 h, when >50% of animals in controls are, at a minimum, at the 7$^{th}$ stage of development out of 13 stages within the copepod life-cycle. Like other larval development tests, it is not known whether longer exposure would reveal whether copepods are experiencing a delay in larval development, or an inability to develop any further. Regardless, observed inhibition in larval development will ultimately result in reduced population growth rate overall.

Compared to other marine copepods, *A. sinjiensis* is one of the most sensitive species to nickel exposure. This could be due to differences in the test method used in the current study compared with methods used for other copepod species, including factors such as test endpoint, diet and the type of algae and its concentration. In the present study, algae were centrifuged and resuspended in natural filtered seawater to remove nutrient rich culture media, while, in other studies, algae were not rinsed of culture media prior to addition to test solutions (Bielmyer et al., 2006; Gorbi et al., 2012; Mohammed et al., 2010; Tlili et al., 2015; Zhou et al., 2016). This can reduce the bioavailability and subsequent toxicity of nickel because algal culture media contains ligands such as ethylenediaminetetraacetic acid (EDTA) that can bind metals. The algal cell densities used in our tests were also between 1-3 orders of magnitude lower than previous studies (Bielmyer et al., 2006; Mohammed et al., 2010; Zhou et al. 2016). In those studies, where a higher algal cell density was used, a

greater depletion of dissolved metal would occur (Franklin et al., 2002), which may lead to a decrease in metal availability, reduced metal uptake, and lower toxicity if the metal then becomes less available to the copepod. Lower cell densities are more environmentally-relevant, and it is evident that the nutritional status of the copepods was not reduced because of the successful development observed in the control treatments (60-80% larval development ratio in controls, in all tests).

In comparison to the snail also tested in this study, the copepod was 12 times more sensitive (based on EC10 values). Algae provided as a food source to the snails during exposure were not washed prior to addition to the test solutions. It is possible, as discussed above, that ligands in the culture media could reduce the bioavailability and toxicity of nickel to the snails. However, the concentrations of total and dissolved metals were measured throughout, and for the snail tests, the proportion of dissolved nickel was >95% (data not shown). Therefore, it is unlikely that the culture media from the algae altered the bioavailability and toxicity of nickel to snails.

### 4.4.3. Nickel in the aquatic environment

Concentrations of nickel in surface marine waters are typically <5 µg Ni/L (Apte et al., 2006, DeForest and Schlekat, 2012). However, at more contaminated sites, nickel concentrations have been reported in the range of 15 - 2000 µg Ni/L (Denkhaus and Salnikow, 2002; Eisler, 1998; Pyle and Couture, 2012). The nickel concentrations used in this study are environmentally relevant, and significant effects were observed at 5 – 67 µg Ni/L for the copepod, snail and barnacle. If these organisms were exposed to elevated concentrations of nickel in the environment, adverse effects could occur. This suggests that nickel could pose a risk to tropical biota in coastal waters.

In natural systems, metals may be directly absorbed by organisms from the water column or assimilated via dietary exposure routes. Toxicity will depend on whether the metal is biologically available, its cellular mode of action and the rate with which it gets eliminated from the body (Luoma, 1983). In this study, algae were added to all three tests as a food

source throughout exposure. In a recent study by Tlili et al. (2015), the adult copepods *Pseudodiaptomus marinus* were exposed for 7 days to nickel by water exposure only and through dietary exposure via an alga *Isochrysis galbana* which was dosed with sub-lethal concentrations of nickel. It was shown that *I. galbana* had a higher uptake rate than the copepod (0.51 µg/L/d compared to 0.17 µg/L/d), and that the copepod accumulated more nickel through dietary exposure (0.17 µg/L/d), than a water-only exposure (0.15 µg/L/d) (Tlili et al., 2015). According to Tlili et al. (2015), the majority of nickel accumulated by microalgae is distributed as soluble substances which may be easily assimilated by higher trophic organisms which feed on algae. In the snail, barnacle and copepod tests, >95% of total nickel was in the dissolved phase, therefore it can be assumed that exposure was predominately via the water route.

### 4.4.4. Toxicity of copper to gastropod and crustaceans

For all three species, copper was more toxic than nickel and toxicity estimates presented here are similar to those reported previously by Trenfield et al., (2016), van Dam et al., (2016) and Binet et al., (2019). Toxicity tests with the snail, barnacle and copepod required the addition of microalgae as a food source over the exposure period. As discussed in section 4.4.2, addition of a food source in chronic exposures is important in ensuring optimal health of the test organisms, starvation over the sensitive early life-stage would compound metal toxicity. However, addition of algae can also result in depletion of dissolved metal concentrations as metal is adsorbed/absorbed by algae (Trenfield et al., 2016, van Dam et al., 2016, Binet et al., 2019). In all three toxicity tests there was up to a 67% loss in dissolved copper concentrations over the exposure period, however this was also similar to what has been reported for the snail (Trenfield et al., 2016), barnacle (van Dam et al., 2016) and copepod Binet et al., (2019) in past studies.

## 4.5. Conclusion

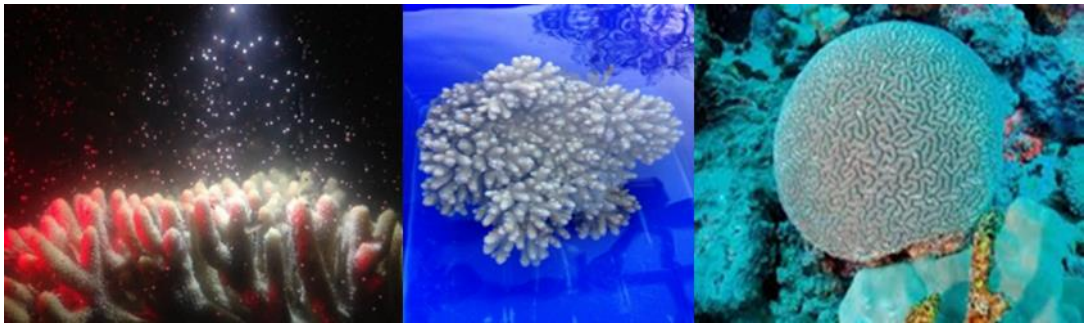
The development of ecologically-relevant risk assessment tools for nickel in tropical marine environments is hindered due to the paucity of data on the effects of nickel on key tropical

species. This chapter has reported the toxicity of nickel to one gastropod and two crustaceans, relevant to tropical Asia-Pacific. These test organisms are ideal species to include in WQG development for nickel due to their sensitivity to the metal, in particular the copepod. It is anticipated that the data presented here will contribute to the development of an ecologically-relevant WQG for nickel in tropical marine waters. Given the potential concentrations of nickel in the environment in close surrounds to nickel mines and facilities, there is potential risk of nickel toxicity to tropical marine organisms in these locations. This chapter has addressed data gaps identified in Chapter 2. Another key taxa for which nickel toxicity data are limited includes corals, which is addressed in the following chapters.

# 5. INHIBITION IN CORAL FERTILISATION FOLLOWING EXPOSURE TO NICKEL AND COPPER

## Context statement

Chapter 2 identified that data are lacking for keystone species such as scleractinian corals, which create the complex structural reef habitats that support many other marine species. The data reported in this chapter builds on toxicity data for other tropical marine species presented in the preceding chapters. This chapter has investigated the toxicity of nickel on fertilisation success in three species of scleractinian corals: *Acropora aspera*, *Acropora digitifera* and *Platygyra daedalea*. In the literature, more data are available on the effects of copper on coral fertilisation, so to allow for comparisons with past studies, the toxicity of copper to *A. aspera* and *P. daedalea* was also determined. This chapter has been adapted from the following publication[4].



[4]Gissi, F., Stauber, J., Reichelt-brushett, A., Harrison, P.L., Jolley, D.F. (2017). Inhibition in fertilisation of coral gametes following exposure to nickel and copper. Ecotoxicology and Environmental Safety 145, 32–41. doi:10.1016/j.ecoenv.2017.07.009. I conducted fertilisation experiments (with assistance from J. Stauber, A. Reichelt-Brushett and P.L. Harrison), I analysed metal sub-samples, completed statistical analysis, and prepared the manuscript which was reviewed by all co-authors.

## 5.1. Introduction

Coral reefs throughout Southeast Asia are considered to be under the greatest threat of decline due to anthropogenic climate change, physical destruction, overfishing, pollution and sedimentation (Burke et al., 2012; Wilkinson et al., 2016). There have been limited field studies investigating the impact of mining activities on coral reefs and tropical marine biota. In a coral reef lagoon in New Caledonia, concentrations of suspended particulate nickel were reported to be >7000 mg/kg. It was believed this was a result from natural and mining-related terrigenous inputs (Fernandez et al., 2006). While this nickel was in particulate form, and likely to be less available for accumulation by biota, the increased and long-term input of this terrigenous material may increase the exchangeable fraction of metal from the sediment to the water, which may become bioavailable and be absorbed by local organisms (Fernandez et al., 2006). Dissolved nickel concentrations around the Indo-Pacific have been reported to be <4 µg/L (Appendix D, Table D1) (Mokhtar et al., 2012; Srichandan et al., 2016), although at polluted sites around the globe, nickel concentrations can reach 2000 µg/L (Eisler, 1998; Pyle and Couture, 2012).

Studies around New Caledonia have shown that key taxa including cephalopods (Bustamante et al., 2000), bivalves (Hedouin et al., 2009) and ascidians (Monniot et al., 1994) have elevated concentrations of nickel in their tissues in areas of increased nickel mining activity. No data were available to assess nickel accumulation in corals in response to mining. However, research within the Asia-Pacific region has shown that corals accumulate other metals in response to mining activities including zinc and lead in Papua New Guinea (Fallon et al., 2002), copper, manganese and zinc in the Philippines (David, 2003) and copper, zinc, chromium, cobalt and molybdenum in Thailand (Howard and Brown, 1987). Howard and Brown (1987) also reported nickel concentrations of 44 µg/g in the tissues of corals adjacent to a tin smelter. A study investigating trace metals in corals from the Great Barrier Reef, Qld, Australia, reported background concentrations of nickel in scleractinian corals ranging from <0.03-0.56 µg/g (Denton and Burdon-Jones, 1986). A

recent study by Hedouin et al. (2016a) used laboratory-based experiments to assess the bioaccumulation of nickel in the scleractinian coral, *Stylophora pistillata* from New Caledonia. Following a 14 day-exposure, results showed that *S. pistillata* could efficiently bioaccumulate nickel within zooxanthellae and host tissues (Hédouin et al., 2016a). While these are useful data on the accumulation of nickel in coral tissues, studies so far have not provided information on the potential toxicity of nickel to corals.

Chapter 2 showed that there are limited high quality data on the toxicity of nickel to key tropical marine species, including corals. Two studies have assessed the impact of nickel on 5-h fertilisation success in corals (Reichelt-Brushett and Harrison, 2005; Reichelt-Brushett and Hudspith, 2016), however, only the latter study used measured nickel concentrations. Previous studies with corals have shown that their response to metals can vary within the same species, so additional data are needed to quantify interspecies variability (Reichelt-Brushett and Harrison, 2005).

The aim of this study was to address the data gaps regarding the lack of coral-specific toxicity information. This chapter investigated the toxicity of nickel and copper to three species of corals with widespread distribution in Indo-Pacific reefs, including the brain coral, *P. daedalea* (tested previously by Reichelt-Brushett and Hudspith (2016)) of the Merulinidae family, and two species of branching coral from the Acroporidae family, *Acropora aspera* and *Acorpora digitifera*. Corals spawn once per year and so this limits our ability to repeat toxicity tests using the fertilisation endpoint. All three species used in this study were hermaphrodite, broadcast spawning corals that release sperm and eggs into the water column for external fertilisation (Veron 1986). Fertilisation success is an ecologically-relevant endpoint to use when assessing metal toxicity, because during external fertilisation gametes are in direct contact with the water column and may be exposed to trace metals (Hudspith et al., 2017). In addition, past studies have shown that coral gametes are sensitive to metals (Hédouin and Gates, 2013; Negri and Heyward, 2001; Reichelt-Brushett and Harrison, 1999; Reichelt-

Brushett and Harrison, 2005; Reichelt-Brushett and Hudspith, 2016; Victor and Richmond, 2005).

## 5.2. Methods

### 5.2.1. General laboratory techniques and reagents

All glassware and plastic containers used in the tests were acid-washed as described in Chapter 3 (Section 3.2.1), then soaked in natural seawater for at least 24 h.

All metal stock solutions were made volumetrically using high purity water. Copper stock solutions of 5 and 100 mg Cu/L (acidified to 0.1% HCl (Tracepur, Merck)) and a nickel stock solution of 100 mg/L (acidified to 0.01% HCl) were also prepared (Chapter 3, Section 3.2.1).

### 5.2.2. Toxicity tests with corals – 5-h fertilisation success

Toxicity test methods followed those described in Reichelt-Brushett and Harrison (1999, 2005), and Reichelt-Brushett and Hudspith (2016). Toxicity tests in this chapter were conducted on Heron Island, southern Great Barrier Reef Marine Park, Australia, during a mass spawning event in November 2015. Test parameters and conditions are shown in Table 5.1.

Gravid coral colonies, with pigmented mature eggs (Harrison et al., 1984), were collected from the reef flat and placed in outdoor aquaria with rapid flow-through natural seawater, approximately 2-3 days before spawning was predicted to occur. Each individual colony was kept in its own tank to ensure identification and separation of egg-sperm bundles from specific colonies. Three species of corals were collected and tested including the brain coral *P. daedalea* and two branching corals, *A. aspera* and *A. digitifera*.

Table 5.1. Toxicity test conditions for 5-h fertilisation tests with corals

| **Toxicity test parameters** | |
|---|---|
| Temperature (°C) | 25 ± 2 |
| pH | 8.1 ± 0.1 |
| Salinity (‰) | 34 ± 1 |
| Conductivity (mS/cm) | 51 ± 1 |
| Dissolved oxygen (mg/L) | >8 |
| Light | Ambient natural light |
| Test type | Static no renewal |
| Test duration | 5 h (+ 30 min of separate egg and sperm exposure) |
| Test chamber | 20 mL glass scintillation vials |
| Test solution volume | 20 mL |
| Age of test organism | Gametes |
| Initial spermatozoa density | 2 x$10^6$/mL |
| Initial no. eggs | ~100 |
| No. replicate chambers per treatment | 5 |
| Control/diluent water | Natural, sperm free seawater |
| Test endpoint | Fertilisation success |
| Test acceptability | >80% fertilisation in controls |

On the afternoon prior to spawning, sperm-free seawater (unfiltered) was collected from the reef-flat in seawater-soaked 20-L polyethylene containers. This seawater was used to make treatment solutions in clean 500-mL polycarbonate containers by adding the required volume of metal stock to achieve the desired nominal concentration. Sub-samples were taken from these bulk treatment solutions for analysis of total and dissolved metals (one sample per treatment) (Chapter 3, Section 3.2.4). Physico-chemical parameters (including pH, salinity, dissolved oxygen and temperature) of seawater and treatment solutions were recorded (Chapter 3, Section 3.2.1).

Spawning occurred during two nights with *P. daedalea* colonies spawning on the November 4, and *Acropora* colonies spawning on the November 5, 2015 (7 and 8 nights after the October full moon, respectively). The nominal nickel concentration values for *P. daedalea* were 100, 500, 1000, 2500, 5000 µg Ni/L, and for *Acropora* species were 300, 1000, 2500, 5000, 10000 µg Ni/L. The concentrations for copper were the same for both species tested: 10, 20, 40, 80 µg Cu/L (nominal).

Immediately after spawning, egg sperm bundles were collected from each colony and were separated by gently washing with seawater and a 120-µm plankton mesh filter was used to

separate the eggs and sperm. Test crosses were set up to ensure gametes from selected colonies were able to cross-fertilise successfully, before being used in the fertilisation experiments.

Separate experiments were performed for each metal (nickel and copper). Four to five metal treatments were set up alongside a control (seawater), with five replicates per treatment. Bulk metal treatment solutions were made at 2x the required concentration to account for dilution when added to the test vials. One set of 20 mL test vials contained 4x concentrated sperm in 5 mL of seawater, to account for dilution following addition of egg and treatment solution. The density of concentrated spermatozoa was determined using a haemocytometer to calculate the volume required to achieve a final concentration of ~2 $x10^6$ /mL in 20 mL of seawater (following addition of egg and treatment solutions). This concentration ensures ~80% fertilisation success in control treatments (Harrison and Ward, 2001; Reichelt-Brushett and Harrison, 1999). In another set of 20 mL vials, ~100 eggs were added to 5 mL of seawater. Eggs were added, by glass pipette, to mini 48-well plates and photographed. These photos were counted later to determine the exact number of eggs added to each vial. Each treatment included five vials with sperm and five vials with eggs. Eggs and sperm were exposed separately by adding 5 mL of the metal solution to each vial to achieve the target nominal concentration. Additional replicates were set up for physico-chemical measurements. All vials were capped and allowed to incubate at room temperature (~25°C) for 30 minutes. After this time, the 10 mL of dosed spermatozoa solution was transferred into the 10 mL of dosed egg solution, vials were capped again, placed in large zip-lock bags and placed in 20 L tubs in the outdoor aquaria with strong water flow and aeration to maintain temperature and to create water movement, providing optimal conditions for successful embryo development (Harrison and Ward, 2001).

An exposure duration of 5 h (excluding 30 minutes of separate egg/sperm exposure) was used, as this has previously been found to be sufficiently long to achieve maximum fertilisation and to reach early embryo stages that indicate successful fertilisation has

occurred (Negri and Heyward, 2001; Reichelt-Brushett and Harrison, 2005). After 5 h, vials were removed, physico-chemical parameters were recorded, one replicate per treatment was selected to sub-sample for total and dissolved metals, and then all vials were fixed with formalin (5 mL of treatment solution was removed from each vial and replaced with 5 mL of 10% formalin). Fertilisation success was observed by counting the number of unfertilised eggs under a stereomicroscope. Unfertilised eggs were counted because these had better structure than the fertilised eggs and enabled a more accurate assessment. The number of fertilised eggs was calculated by subtracting the number of unfertilised eggs from the number of eggs added at t=0. Fertilisation success was expressed as a % of control.

### 5.2.3. Chemical and statistical analyses

Sub-samples for total and dissolved metals in test solutions were sampled, preserved and analysed (Chapter 3, Section 3.2.4). The mean of the dissolved measured values was taken from t=0 and t=5 h for statistical analysis. Statistical analyses were undertaken as per Chapter 3, Section 3.2.5. Models to calculate toxicity estimates were selected based on the AIC value (Appendix D, Table D2).

Dissolved organic carbon (DOC) in seawater (diluent/control water) was sampled and analysed as described in Chapter 3, Section 3.2.4.

## 5.3. Results

### 5.3.1. Quality assurance

Over the 5-h exposure, in all tests, physico-chemical parameters were within acceptable limits (Table 5.1), with measured values of pH 8.1 ± 0.1, salinity 34 ± 0.3‰ and DO 8.1 ± 0.2 mg/L (± standard deviation, (SD)). Temperature was maintained at 25 ± 2°C in the outdoor aquaria.

Background concentrations of metals in the seawater used in all tests were below the limits of detection (LOD, Fe 0.47, Mo 0.68, Mn 0.60, Al 0.51, As 4.4, Ba 0.20, Cd 0.35, Co 0.55, Cr 0.61, Cu 0.80, Ni 2.1, Se 3.5, V 0.69 µg/L), except for Test 2 (*A. digitifera* and *A. aspera*)

where molybdenum and copper were detected at 0.86 and 1.2 µg/L, respectively (Appendix D, Table D3).

Measured dissolved nickel concentrations were within 88-97% of nominal values. The loss of nickel in test vials over 5 h was low, between 0-5% (Appendix D, Table D4). There was no significant difference between total and dissolved nickel, indicating that the nickel in the test solutions was in the dissolved phase (data not shown).

Measured dissolved copper concentrations were within 46-67% of nominal values. This is attributed to the loss of copper in test solutions to the glass vial walls, and potentially due to uptake by the coral gametes over 5 h (Appendix D, Table D4). The difference between total and dissolved copper in test solutions was between 4-23% (data not shown).

The fertilisation success for all three species met acceptability criteria (>80% fertilisation in controls, Table 5.1), with ≥96% fertilisation in control treatments (data not shown).

### 5.3.2. Toxicity of nickel to corals – 5-h fertilisation success

Based on the calculated endpoints and the concentration response curves (Table 5.2, Figure 5.1 A-C), *A. aspera* was the most sensitive to nickel, with a NOEC of <280 µg Ni/L. For the next most sensitive species, *A.digitifera*, fertilisation success was inhibited by 10% at 2000 (1580-2420) µg Ni/L (95% confidence limits, CL). The NOEC for this species was 940 µg Ni/L (Table 5.2). The least sensitive species to nickel was *P. daedalea* with a NOEC of 920 µg Ni/L (Table 5.2). There was a very small but significant effect on fertilisation at concentrations ≥920 ug/L (Figure 5.1 C). The slopes of the nickel concentration response curves for the three species were different, with the slope for *A. digitifera* (Figure 5.1 B) being steep, while for *A. aspera* and *P. daedalea* the slopes were more gradual (Figure 5.1 A, C).

### 5.3.3. Toxicity of copper to corals – 5-h fertilisation success

Copper was more toxic than nickel to coral fertilisation. Based on the EC10 values, *A. aspera* was the more sensitive species, with an EC10 (95% CL) of 5.8 (1.6-10) µg Cu/L

compared to *P. daedalea* at 16 (14-18) µg Cu/L (Table 5.2). At higher percentage effect levels, *P. daedalea* was more sensitive to copper, with an EC50 of 28 (27-30) µg Cu/L compared to *A. aspera* (EC50 of 78 (36-121) µg Cu/L) (Table 5.2), although this value is an extrapolation beyond the highest concentration tested (54 µg Cu/L). This is also shown in Figure 5.2 where the slope of the concentration response curve for *P. daedalea* (Figure 5.2 B) is much steeper than that of *A. aspera* (Figure 5.2 A). In toxicity tests with *A. aspera,* complete inhibition was not observed and at the highest concentration tested, fertilisation success was >40% (Figure 5.2 A). In contrast, the highest concentration tested for *P. daedalea* was 37 µg Cu/L resulting in fertilisation success of <30% (Figure 5.2 B).

Table 5.2. Toxicity of nickel and copper to fertilisation success in corals following a 5-h exposure. Toxicity estimates and NOEC values for measured dissolved (0.45 µm filtered) metal. Values in parentheses are 95% confidence limits. Toxicty estimates (EC5, 10, 50) were calculated using the drc package in R. No observable effect concentration (NOEC) values were calculated using Bonferroni's t-test (2-tailed, $p<0.05$) in Toxcalc.

| | Nickel (µg/L) | | | | Copper (µg/L) | | | |
|---|---|---|---|---|---|---|---|---|
| **Species** | **EC5** | **EC10** | **EC50** | **NOEC** | **EC5** | **EC10** | **EC50** | **NOEC** |
| *Acropora aspera*[a,b] | NC | NC | >9220 | <280 | 3.3 (0.3-6.2) | 5.8 (1.6-10) | 78 (36-121) | <6 |
| *Acropora digitifera*[b] | 1680 (1260-2110) | 2000 (1580-2420) | 4350 (3830-4870) | 940 | NT | NT | NT | NT |
| *Platygyra daedalea*[a] | >4610 | >4610 | >4610 | 920 | 13 (10-15) | 16 (14-18) | 28 (27-30) | 9 |

[a] Weibull model 1.3 used to calculate toxicity estimates. Weibull 1.3 used to calculate toxicity estimates for nickel and *A. aspera*
[b] Weibull model 2.3 used to calculate toxicity estimates. Weibull 2.3 used to calculate toxicity estimates for copper and *A. aspera*
NC, not calculated
NT, not tested

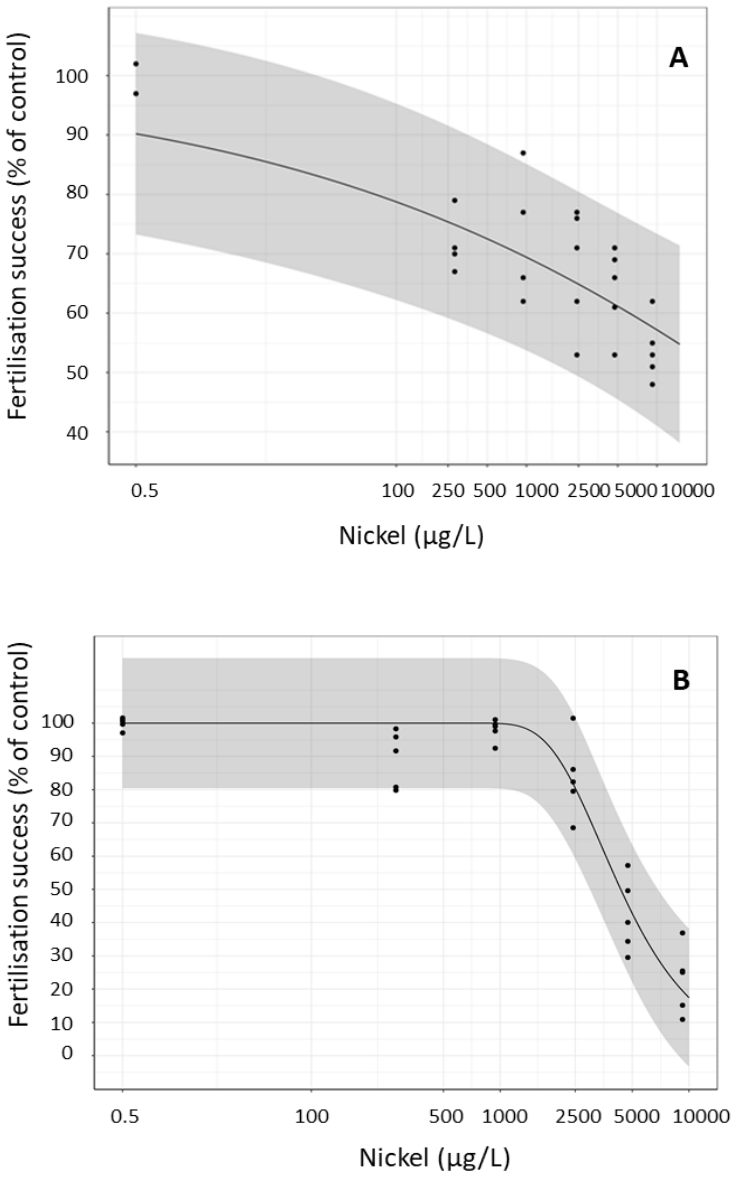



Figure 5.1. Toxicity of nickel to fertilisation success (% of control) in the corals A) *Acropora aspera*, B) *Acropora digitifera* and C) *Platygyra daedalea*. The grey ribbon shows the 95% prediction interval of the model (black line). Each point represents one replicate. Data are from one individual toxicity test. Nickel values are dissolved (0.45 µm filtered) measured concentrations; control concentration was set to 0.5 µg Ni/L, which is approximately half the LOD for nickel by ICP-AES. Note the different scales on the x- and y- axes.

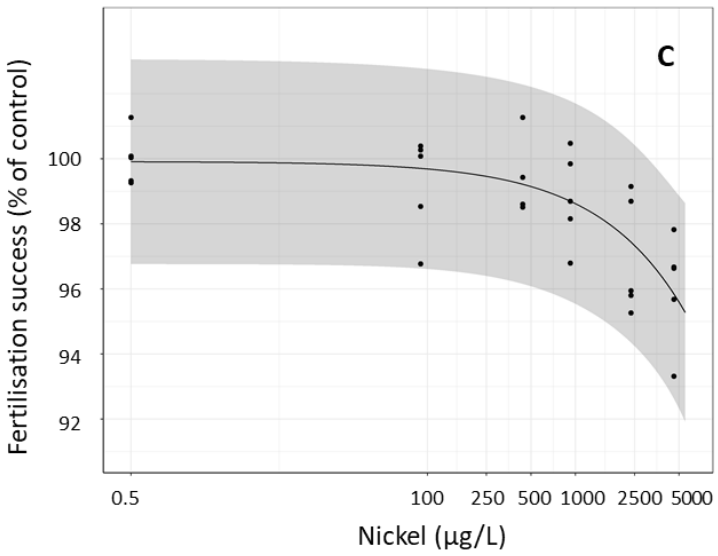



Figure 5.1. Continued.

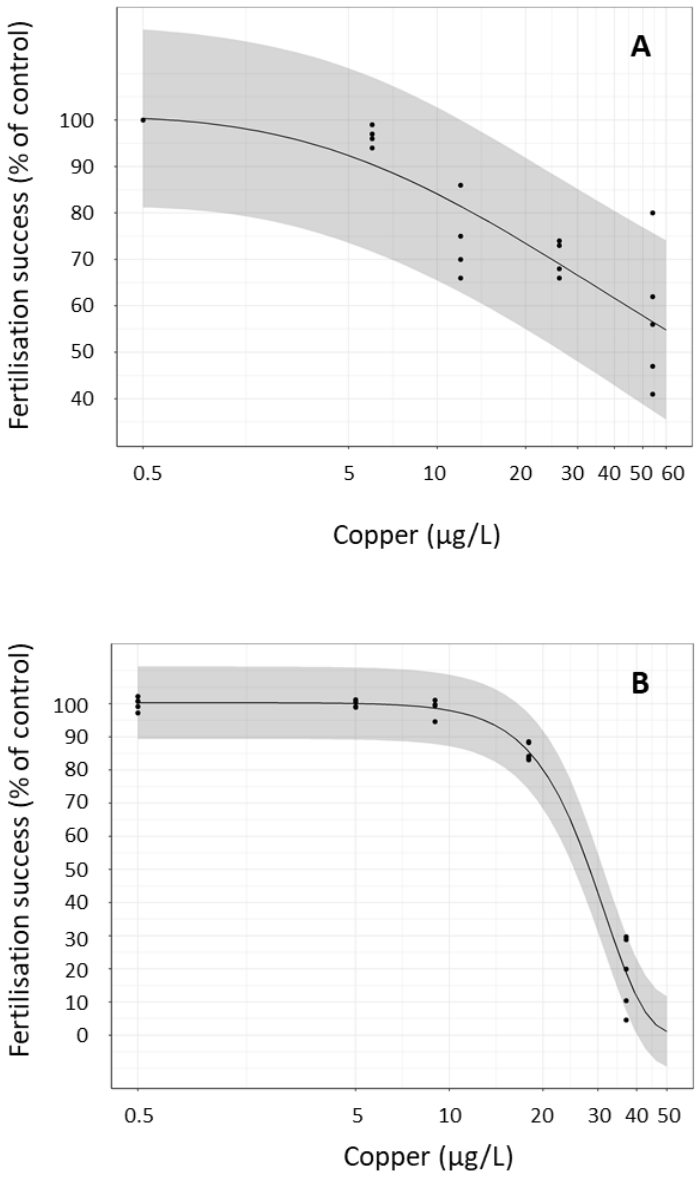



Figure 5.2. Toxicity of copper to fertilisation success (% of control) in the corals A) *Acropora aspera*, B) *Platygyra daedalea*. The grey ribbon shows the 95% prediction interval of the model (black line). Each point represents one replicate. Data are from one individual toxicity test. Copper values are dissolved (0.45 µm filtered) measured concentrations; control concentration was set to 0.5 µg Cu/L, which is approximately half the LOD for copper by ICP-AES. Note the different scales on the y axes.

## 5.4. Discussion

Many corals live within a narrow range of water quality and temperature conditions and are very sensitive to changes in their environment (Harrison and Booth, 2007). Chemical communication is key in mediating processes such as reproduction and settlement which are central to the survival and persistence of coral reefs (Harrison and Wallace, 1990; Peters et al., 1997). Water quality changes may occur as a result of anthropogenic inputs and this can disrupt key chemical interactions among reef organisms (Bielmyer et al., 2010; Peters et al., 1997). Coral gametes and larvae are particularly sensitive to changes in water quality because they are released into the environment to fertilise and undergo development where they are potentially in direct contact with anthropogenic contaminants (Reichelt-Brushett and Hudspith, 2016).

The development of ecologically-relevant risk assessment tools requires toxicity data for key tropical marine species such as corals. However, there are few published toxicity data for the effect of nickel on fertilisation success in corals (Reichelt-Brushett and Harrison, 2005; Reichelt-Brushett and Hudspith, 2016). In the present study, the toxicity of nickel and copper on fertilisation success has been assessed for three different species of scleractinian corals, two from the Acroporidae family (*A. aspera* and *A. digitifera*) and one from the Merulinidae family of brain corals (*P. daedalea*). *Acropora aspera* is found on upper reef slopes and lagoons, *A. digitifera* is found in shallow reef environments and *P. daedalea* is found in all areas of reef environments, commonly on back reef margins (Aeby et al., 2014a, 2014b; DeVantier et al., 2014). The International Union for Conservation of Nature (IUCN) Redlist classifies *A. aspera* as a vulnerable species and *A. digitifera* as a near-threatened species (Aeby et al., 2014a, 2014b). *Platygyra daedalea* is currently listed as “Least Concern” (DeVantier et al., 2014).

### 5.4.1. Toxicity of nickel to coral fertilisation success

The effect of nickel on the 5-h fertilisation success to corals varied among species, with *A. aspera* the most sensitive, followed by *A. digitifera* and *P. daedalea*. The first species tested

was *P. daedalea* and the concentration range selected for this test was based on a previous study with the same species also at Heron Island (Reichelt-Brushett and Hudspith, 2016). In the present study the EC50 was estimated to be >4610 µg Ni/L. This differs to the results of Reichelt-Brushett and Hudspith (2016), who reported an EC50 of 1420 (1160-1800) µg Ni/L. Reichelt-Brushett and Harrison (2005) investigated the toxicity of nickel to the coral *G. aspera,* over two spawning events over two consecutive years, at different locations. In the first experiment of that study, conducted at Magnetic Island (Qld, Australia), they found that fertilisation success of *G. aspera* gametes was >83% in all treatments, including the highest nickel treatment of 2000 µg Ni/L. The following year, experiments were completed at One Tree Island (Qld, Australia) and a significantly lower fertilisation rate occurred in nickel treatments ≥ 100 µg Ni/L. At the highest concentration tested (2000 µg Ni/L), fertilisation success was 60%, however these estimates were based on nominal nickel concentrations only. That study demonstrated that the response of coral fertilisation to metal exposure can vary within the same species between locations. In addition, pre-exposure may be an important factor to consider with respect to location of coral colonies that are selected for toxicity testing. Magnetic Island is close to Townsville Harbour, 8 km from the coast and close to anthropogenic contaminant sources and corals there were found to be less sensitive than those from One Tree Island which is located in the relatively pristine Southern Great Barrier Reef Marine Park, 96 km offshore from the coast (Reichelt-Brushett and Harrison, 2005).

In the present study, on the second night of spawning a higher concentration range of nickel was selected to ensure the full response would be captured. *Acropora aspera* was the most sensitive species with a NOEC value of <280 µg Ni/L, however, EC5 and EC10 values could not reliably be calculated because there were not two or more concentrations tested in between the control and the NOEC (Figure 5.1 A). *Acropora digitifera* was the next most sensitive species to nickel, with a NOEC value of 940 µg Ni/L and an EC10 of 2000 µg Ni/L (Table 5.2). This is the first report on the toxicity of nickel to any *Acropora* species. Given

that the genus *Acropora* is the most widespread and contains the largest number of species of reef-building corals (Wallace, 1999), including data for *Acropora* species increases the confidence in nickel ecotoxicity thresholds that are applied to tropical marine ecosystems.

From the data reported in this study and two other studies investigating the effect of nickel on coral fertilisation (Table 5.3), it is evident that sensitivity of coral gametes to nickel is variable between species, but also variation exists within populations of one species. Only one additional study has investigated the toxicity of nickel to early life stages of coral (Goh, 1991). Goh (1991) studied the effect of nickel on larval survival and settlement in the coral *Pocillopora damicornis*, measuring effects during a recovery period following exposure to nickel for 12-96 h. The LC50 after a 40-h recovery period was >9000 µg Ni/L. Settlement was a more sensitive endpoint, with significantly reduced settlement rates after a 9-day recovery from exposure to 1000 µg Ni/L (Goh, 1991). It is difficult to make direct comparisons with the results obtained in the present study and those of Goh (1991) because *P. damicornis* employs different reproductive strategies than corals of the genus *Acropora* or *Platygyra* which are broadcast spawners that release eggs and sperm into the water column (Harrison and Wallace, 1990). *Pocillopora damicornis* broods sexually or asexually generated larvae which are then released into the sea (Ayre et al., 1997). Additionally, different types of exposures, endpoints and the age of the test organism were used among the studies. In general, it appears that early life stages of corals may be relatively insensitive to nickel, with most effects observed above 1000 µg Ni/L.

There are few studies that report the concentrations of nickel in seawater around the Indo-Pacific region (Appendix D, Table D1), although based on limited information, concentrations of nickel in seawater there are typically <4 µg/L (Mokhtar et al., 2012; Srichandan et al., 2016) and globally in contaminated coastal areas, concentrations of nickel have been reported between 50-2000 µg/L (Eisler, 1998; Pyle and Couture, 2012). Elevated concentrations of nickel of up to 7000 mg/kg have been detected in marine sediments around New Caledonia (Fernandez et al., 2006; Hedouin et al., 2009). In this study, toxic

effects on coral fertilisation occurred at concentrations well above environmentally-relevant concentrations for nickel in seawater around the Indo-Pacific (Appendix D, Table D1). However, at very contaminated sites, it is possible that fertilisation of coral gametes could be impaired at high nickel concentrations.

### 5.4.2. Toxicity of copper to coral fertilisation success

Copper is more toxic to coral fertilisation than nickel. The response to copper exposure is variable between species, with effects on fertilisation success between 11-261 µg Cu/L (based on EC50 values, Table 5.3). The least sensitive species to copper is a soft coral, *Lobophytum compactum* (EC50 261 µg Cu/L) (Reichelt-Brushett and Michalek-Wagner, 2005). This is the only toxicity data available for a soft coral. The EC50 values for hard corals range from 11-145 µg Cu/L (Table 5.3).

The results in this study for *P. daedalea* (EC50 28 µg Cu/L) are similar to that of Reichelt-Brushett and Hudspith (2016) who estimated an EC50 of 33 µg Cu/L. However the EC10 values differ; 16 µg Cu/L in this study, compared to 1.4 µg Cu/L estimated by Reichelt-Brushett and Hudspith (2016). In a more recent study by Hudspith et al. (2017), the response of *P. daedalea* to copper was different again, with the EC50 estimated as 73 µg Cu/L, despite use of the same methods and study location (Heron Island) as that used by Reichelt-Brushett and Hudspith (2016).

Several other studies have assessed copper toxicity on fertilisation success in brain corals (Table 5.3). For corals of the genus *Goniastrea,* toxicity estimates for 50% inhibition in fertilisation ranged from 14.5-25 µg Cu/L (Reichelt-Brushett and Harrison, 1999; Reichelt-Brushett and Harrison, 2005). Heyward (1988) reported NOEC values of ~10 µg Cu/L for the corals *P. ryukyuensis* and *Favites chinensis*. NOEC values in other studies ranged from 2 – 13 µg Cu/L, and our result (NOEC value for *P. daedalea* of 9 µg Cu/L) was in good agreement with these studies. Conversely, Kwok et al. (2016) found the coral *P. acuta* to be much less sensitive to copper with fertilisation success inhibited by 50% (EC50) between 92 - 145 µg Cu/L (based on nominal concentrations). This response may be due to the coral

colonies being pre-exposed to elevated concentrations of copper in seawater around Hong Kong (Kwok et al., 2016).

There are more data available on the effect of copper on fertilisation success in corals from the Acroporidae family (Table 5.3). Only one study for the coral *M. capitata* provided EC10 estimates of 9 - 15 µg Cu/L over three consecutive tests (Hédouin and Gates, 2013). This is similar to our study where fertilisation success in *A. aspera* was inhibited by 10% at 5.8 (1.6-10) µg Cu/L. The EC50 estimates for Acroporidae corals ranged from 15 – 75 µg Cu/L (Hédouin and Gates, 2013; Negri and Heyward, 2001; Puisay et al., 2015; Reichelt-Brushett and Harrison, 2005; Victor and Richmond, 2005) (Table 5.3). The results from our study for *A. aspera* (EC50 78 µg Cu/L) were similar to those of previous studies and are most similar to results obtained by Puisay et al. (2015) for *A. cytherea* and *A. pulchra* with EC50 values of 69 and 75 µg Cu/L, respectively. Caution must be taken when making comparisons with other studies, particularly where the test methods vary (Hudspith et al., 2017). This is the case with the studies by Puisay et al. (2015) and Victor and Richmond (2005), who exposed gametes to contaminants simultaneously, unlike in our study and that of Reichelt-Brushett and Harrison (1999, 2005), Hedouin and Gates (2013), Reichelt-Brushett and Hudspith (2016) and Hudspith et al. (2017), among others, where the test methods exposed egg and sperm separately prior to combining and monitoring fertilisation success after a further exposure for 3-5 h.

### 5.4.3. Variability in sensitivity to metals between different coral endpoints

The results presented in this chapter and other published data show that there is considerable variability in the sensitivity of corals to metals (Table 5.3). It is difficult to distinguish patterns among different families or groups of corals due to the lack of sufficient data and the use of different test methods, however, in general, it appears that Acroporidae corals are more sensitive to nickel than brain corals, and the reverse is found for copper. The sensitivity of a species to a metal contaminant is both species- and metal-specific. The differences in toxicity of metals to coral gametes could be due to different gamete

ultrastructure and morphology, biochemical processes or underlying tolerances to stressors between species (Harrison, 1990; Hudspith et al., 2017). To understand these differences in sensitivities, further research into the mechanisms of metal toxicity to external invertebrate fertilisation is required (Hudspith et al., 2017).

Some previous studies have assessed the effect of copper on coral embryo viability, larval survival, motility or swimming activity and metamorphosis or settlement (Table 5.3). In this study, attempts were made to assess the effect of nickel and copper on embryo viability by continuing exposure of gametes from fertilisation up to 10 h, and also larval survival from fertilisation up to 72 h. This was only tested with *P. daedalea* for nickel. In the 10-h exposure, no significant response was observed (data not shown). There is only one other published study that has also continued exposure of embryos to copper following fertilisation. Victor and Richmond (2005) exposed the gametes of *A. surculosa* to copper and found that between 5 and 12 h, the EC50 decreased nearly fourfold from 45 µg Cu/L to 11 µg Cu/L. In the present study, in experiments where larval survival was monitored from fertilisation up to 72 h, no concentration-response relationship was observed, although survival in controls was >65% (Appendix D, Table D5, Figure D1). Investigating this endpoint further would require method development, particularly around the issue of renewing treatment solutions to maintain optimal water quality conditions. Based on the literature, larval survival does not appear to be more sensitive to copper than fertilisation success, with EC50 values ranging from 80-198 µg Cu/L. Settlement may be a slightly more sensitive endpoint with 50% effects observed in *Acropora* species between 26-110 µg Cu/L (Negri and Heyward, 2001; Negri and Hoogenboom, 2011; Reichelt-Brushett and Harrison, 2000). The motility of coral larvae appears to be a more sensitive endpoint with EC50 values ranging from 22-48 µg Cu/L (Kwok et al., 2016; Reichelt-Brushett and Harrison, 2004), suggesting that further investigation of this endpoint's sensitivity and reproducibility is desirable.

### 5.4.4. Toxicity testing with coral gametes

This study and the recent studies of Reichelt-Brushett and Hudspith (2016) and Hudspith et al. (2017) demonstrated that the same species of coral from the same location may show variations in sensitivities to metals. It is unlikely that these differences are due to the experimental approach used as the standard coral fertilisation methods, originally based on those of Reichelt-Brushett and Harrison (1999, 2005) were employed in all three studies. In corals, the gametogenesis cycle occurs over approximately five to nine months (Harrison and Wallace, 1990). Changes in environmental conditions due to natural or anthropogenic stressors (e.g. increase water temperature, coral bleaching, pollution, etc.) can influence the reproductive cycles and viability of coral gametes (Harrison and Ward, 2001; Hudspith et al., 2017; Ward et al., 2002). Past studies have demonstrated that the plasticity of coral gametes (e.g. egg size, no. of eggs per bundle) is linked to the environmental conditions associated with the parent colonies, and to the month of spawning (Hédouin and Gates, 2013; Padilla-Gamiño and Gates, 2012; Padilla-Gamiño et al., 2011). Phenotypic variability in coral gametes may enable coral species to cope with and adjust to changes in environmental conditions and stressors (Hédouin and Gates, 2013). If there is inherent variability in coral gametes, then their susceptibility to metal exposure is likely to vary temporally and between individuals within the same species (Hudspith et al., 2017). Future studies should determine the variability in sensitivity of individual colonies over time. This would require tagging colonies and testing the gametes from the same colonies over multiple years or spawning events.

An additional factor that could influence the variability of gamete sensitivity to metals is sperm concentration. Fertilisation success is determined by sperm concentration, with both too little and too many sperm resulting in decreased fertilisation (Marshall, 2006). Field and laboratory studies have shown that in several species of scleractinian corals, optimal fertilisation success (i.e. ~ 100% fertilisation) occurs between $10^5$ - $10^6$ sperm/mL (Oliver and Babcock, 1992; Willis et al. 1997). In this study and many previous studies investigating

toxicity of metals to coral fertilisation, the sperm concentration (2 x$10^6$ sperm/mL) used in toxicity tests exceeded this concentration. High concentrations of sperm can result in polyspermy that can reduce fertilisation success. Exposure to metals could therefore potentially reduce sperm numbers, thereby increasing fertilisation because polyspermy is reduced (Marshall, 2006). Metal toxicants can affect different aspects of fertilisation including sperm survival, inhibition of the proportion of egg to sperm contact or by impeding polyspermy blocks in eggs (Hudspith et al., 2017; Marshall, 2006). To investigate the effects of sperm concentration on sensitivity to toxicants it has been proposed that ecotoxicological studies on coral fertilisation should utilise a range of ecologically relevant sperm concentrations (Jones et al., 2015; Marshall, 2006). The aim of the present study was to compare our results with previous studies and so the same methods were used, including sperm concentration. It has been shown that at this sperm concentration (2 x$10^6$ sperm/mL) fertilisation success in controls (unexposed gametes) is ≥80% suggesting optimal conditions (Hudspith et al., 2017; Reichelt-Brushett and Hudspith, 2016; Reichelt-Brushett and Harrison, 2005, 1999).

The available coral fertilisation toxicity data are difficult to compare directly across multiple studies due to the differences in methodologies used. The sensitivity of a species to a metal contaminant can also be influenced by the experimental design. The majority of studies on the toxicity of metals to coral fertilisation use standardized methods originally described in Reichelt-Brushett and Harrison (1999, 2005), and the present study followed the same protocol in order to verify and compare our results with previous studies. A recent review by Hudspith et al. (2017) has highlighted the need to standardise toxicity testing methods for fertilisation assays with broadcast spawning invertebrates. Key issues that need to be considered to harmonise methods include exposing eggs and sperm together or separately, the egg-to-sperm ratio, the egg/sperm densities, the type of diluent/control water used in the test and the duration of exposure (Hudspith et al., 2017).

Table 5.3. The effect of nickel and copper on corals. Table modified from Hudspith et al. (2017). Toxicity estimates, and NOEC values presented as metal concentration in µg/L. Values in parentheses are 95% confidence limits or ± standard error.

| Species | Endpoint | Metal Salt | Diluent[a] | EC10 | EC50 | NOEC | Exposure characteristics[b] | Study |
|---|---|---|---|---|---|---|---|---|
| **Nickel** | | | | | | | | |
| ***Acroporidae*** | | | | | | | | |
| *Acropora aspera* | Fertilisation success | $NiCl_2.6H_2O$ | SW | NC | >9220 | <280 | (Gametes 30 min) +5 h | This study |
| *A. digitifera* | | | | 2000 (1580-2420) | 4350 (3830-4870) | 940 | | |
| ***Merulinidae*** | | | | | | | | |
| *Platygyra daedalea* | Fertilisation success | $NiCl_2.6H_2O$ | SW | >4610 | >4610 | 920 | (Gametes 30 min) +5 h | This study |
| *P. daedalea* | Fertilisation success | $NiCl_2$ | SW | NR | 1420 (1160-1800) | NR | (Gametes 30 min) +5 h | Reichelt-Brushett and Hudspith (2016) |
| *Goniastrea aspera* | Fertilisation success | $NiCl_2$ | SW | NR | >2000 | NR | (Gametes 30 min) +5 h | Reichelt-Brushett and Harrison (2005) |
| ***Pocilloporidae*** | | | | | | | | |
| *Pocillopora damicornis* | Larval settlement and survival | $NiCl_2.6H_2O$ | F | NR | 9000[c] | NR | Planulae larvae 12, 24, 48, 96 h | Goh (1991) |
| **Copper** | | | | | | | | |
| ***Acroporidae*** | | | | | | | | |
| *A. aspera* | Fertilisation success | $Cu(SO_4)_2$ | SW | 5.8 (1.6-10) | 78 (36-121) | <6 | (Gametes 30 min) +5 h | This study |
| *A. cytherea* | Fertilisation success | $Cu(HNO_3)_2$ | F | NR | 69.4 | NR | Gametes 4.5 h | Puisay et al. (2015) |
| *A. longicyathus* | Fertilisation success | $CuCl_2$ | SW | NR | 15.2 (12-19.2) | 15.3 | (Gametes 30 min) +5 h | Reichelt-Brushett and Harrison (2005) |
| *A. millepora* | Fertilisation success | $CuCl_2$ | F | NR | 17.4 (± 1.1)[d] | NR | Gametes 4 h | Negri and Heyward (2001) |
| | Larval metamorphosis (settlement) | | | NR | 110 (± 20) | NR | 7 d old larvae 24h | |
| | Larval metamorphosis (settlement) | $CuCl_2$ | F | NR | 26 (± 0.98) | NR | 7 d old larvae, 6 h pre-exposure, + 18 h with CCA[e] | Negri and Hoogenboom (2011) |
| *A. pulchra* | Fertilisation success | | | NR | 75.4 | NR | Gametes 4.5 h | Puisay et al. (2015) |
| *A. surculosa* | Fertilisation success | $CuSO_4$ | F | NR<br>NR | 45.2<br>11.4 | NR<br>NR | Gametes 5 h<br>Gametes 12 h | Victor and Richmond (2005) |

Table 5.3. The effect of nickel and copper on corals. Table modified from Hudspith et al. (2017). Toxicity estimates, and NOEC values presented as metal concentration in µg/L. Values in parentheses are 95% confidence limits or ± standard error.

| Species | Endpoint | Metal Salt | Diluent[a] | EC10 | EC50 | NOEC | Exposure characteristics[b] | Study |
|---|---|---|---|---|---|---|---|---|
| *A. tenuis* | Fertilisation success | $CuCl_2$ | SW | NR | 39.7 (36-43.7) | 33.5 | (Gametes 30 min) +5 h | Reichelt-Brushett and Harrison (2005) |
| | Larval metamorphosis (settlement) | $CuCl_2$ | F | NR | 32 (± 0.86) | NR | 7 d old larvae, 6 h pre-exposure, + 18 h with CCA[e] | Negri and Hoogenboom (2011) |
| | Larval metamorphosis (settlement) | $CuCl_2$ | SW | NR | 35 (32-37) | 20 (nominal) | 5 d old larvae, 48 h | Reichelt-Brushett and Harrison (2000) |
| *A. tumida* | Larval survival | $Cu(SO_4)_2$ | ASW | 5.8 (0.1-690)[d] | 80 (11-590)[d] | NR | Planula larvae, 24 h | Bao et al. (2011) |
| *Montipora capitata* | Fertilisation success | $Cu(HNO_3)_2$ | F | 9-15.1 | 16.6-31.7 | NR | Gametes 3 h | Hédouin and Gates (2013) |
| *M. verrucosa* | Adult coral survival | NR | NR | NR | 48 | NR | NR | Howard et al. (1986), from Negri and Heyward (2001) |
| ***Merulinidae*** | | | | | | | | |
| *G.aspera* | Fertilisation success | $CuCl_2$ | SW | NR | 14.5 | 2 | (Gametes 30 min) +5 h | Reichelt-Brushett and Harrison (1999) |
| | | | | NR | 18.5 (12-19.2) | 12.8 | | Reichelt-Brushett and Harrison (2005) |
| | Larvae survival | $CuCl_2$ | SW | NR | 34 (19-62) | NR | 5 d old larvae, 72 h | Reichelt-Brushett and Harrison (2004) |
| | | | | NR | 82 (54-123) | NR | 6 d old larvae, 72 h | |
| | Larvae motility | | | NR | 22 (21-23) | NR | 4-6 d old larvae, 48 h | |
| *G. retiformis* | Fertilisation success | $CuCl_2$ | SW | NR | 24.7 (15.5-30) | 10 | (Gametes 30 min) +5 h | Reichelt-Brushett and Harrison (2005) |
| *P. daedalea* | Larvae motility | $CuCl_2$ | SW | NR | 36 (33-39) | NR | 4-6 d old larvae, 24 h | Reichelt-Brushett and Harrison (2004) |
| | Fertilisation success | $Cu(SO_4)_2$ | SW | 16 (14-18) | 28 (27-30) | 9 | (Gametes 30 min) +5 h | This study |
| | | $NiCl_2$ | | NR | 73 | NR | | (Hudspith et al., 2017) |
| | | | | 1.4 | 33 (30-37) | NR | | Reichelt-Brushett and Hudspith (2016) |
| *P. acuta* | Fertilisation success | $CuCl_2$ | F | NR | 92.1-145 | NR | (Gametes 30 min) +5 h | Kwok et al. (2016)[f] |
| | Larval survival | | | NR | 102-110 | NR | 4 d old larvae, 48 h | |

Table 5.3. The effect of nickel and copper on corals. Table modified from Hudspith et al. (2017). Toxicity estimates, and NOEC values presented as metal concentration in µg/L. Values in parentheses are 95% confidence limits or ± standard error.

| Species | Endpoint | Metal Salt | Diluent[a] | EC10 | EC50 | NOEC | Exposure characteristics[b] | Study |
|---|---|---|---|---|---|---|---|---|
| | | | | NR | 101-107 | NR | 4 d old larvae, 96 h | |
| | Larvae metamorphosis (settlement) | | | NR | NC, no effects observed up to 200 µg Cu/L | NR | 7 d old larvae, 48 h exposure, 48 h clean seawater for settlement | |
| | Swimming activity | | | NR | 45.4-47.7 | NR | | |
| | Settled larvae growth | | ASW | NR | NC, no effects observed up to 200 µg Cu/L | NR | Newly settled recruits (3 w old), 8 weeks | |
| *P. ryukyuensis* *Favites chinensis* | Fertilisation success | NR | NR | NR | <100 | ~10 | NR | Heyward (1988) from Negri and Heyward (2001) |
| ***Pocilloporidae*** | | | | | | | | |
| *P. damicornis* | Larval survival at 27°C | $CuCl_2$ | F | NR | 198 | NR | 96 h exposure | Hedouin et al. (2016) |
| | at 30°C | | | NR | 141 | NR | | |
| | Adult survival at 24°C | | | NR | 251 | NR | Nubbins, 2-4 cm length | |
| | at 27°C | | | NR | 175 | NR | 96 h exposure | |
| | Larval survival | NR | NR | NR | 87<br>57 | NR | 48 h<br>96 h | Esquivel (1986), from Kwok et al. (2016) |
| ***Alcyoniidae*** | | | | | | | | |
| *Lobophytum compactum* | Fertilisation success | $CuCl_2$ | SW | NR | 261 (208-328) | 69 | (Gametes 30 min) +10 h | Reichelt-Brushett and Michalek-Wagner (2005) |

NR, not reported
NC, not calculated
[a] Diluent: SW = sperm free natural unfiltered seawater, F = filtered natural seawater, ASW = Artificial seawater
[b] Exposure Characteristics: (Gametes 30 min) + 5 h = gametes exposed separately for 30 min then combined for 5 h
Gametes 3, 4.5, 5 h = gametes exposed simultaneously for set time
[c] Toxicity estimate calculated during recovery period
[d] Nominal metal concentrations used
[e] CCA = Crustose coralline algae
[f] Tests conducted between 26-30°C

## 5.5. Conclusion

This chapter has shown that the sensitivity of coral gametes to metals is both species- and metal-specific. Based on the data derived in this study and previous publications, copper is more toxic to coral fertilisation than nickel, however, the dataset for nickel is limited. In general, the data reported in this study are in good agreement with previous studies, however, the sensitivity of coral gametes to metals varies between individuals and spatially. This may be part of the natural variation and plasticity of coral gamete development which may vary from year-to-year depending on the conditions of the adult colony. Further research is required to understand the natural variability in the sensitivity of coral gametes to metals between colonies of the same species, between species, and differences over time and geographical location. Future research should also investigate the toxicity of nickel to different life stages, including coral larvae and adult colonies, and assess how *in situ* coral populations subjected to gradients of contamination respond to metal exposure.

The methodologies applied in this study meet the criteria established by government, industry and regulators (e.g. use of ecologically-relevant endpoints and measured metal concentrations) for the use of high-quality data in WQG development. This study has provided the first data on the toxicity of nickel to fertilisation success in corals of the genus *Acropora*. This, together with the additional data for *P. daedalea,* can contribute to the inclusion of corals in the development of WQGs for nickel in tropical marine waters.

It would be valuable to increase our understanding of how nickel impacts the early stages of larval settlement and metamorphosis and also how nickel interacts with the coral animal and its associated microbiota, including the mutualistic *Symbiodinium*. This is addressed in the following chapters.

# 6. THE EFFECT OF DISSOLVED NICKEL AND COPPER ON THE ADULT CORAL *ACROPORA MURICATA*

## Context Statement

This chapter builds on the findings presented in the previous chapter and presents results on the effects of nickel and copper on the adult life-stage of the common branching coral *Acropora mucricata.* In addition, the chapter reports on the effects of these metals on the coral microbiome. Given the fundamental role that the microbiome plays in host functioning, it is important to consider the effects metals have on both the coral and its microbiome. This chapter has been adapted from the following publication [5].


[5] **Gissi, F**., Reichelt-brushett, A., Chariton, A., Stauber, J.L., Greenfield, P., Humphrey, C., Salmon, M., Stephenson, S., Cresswell, T., Jolley, D.F. The effect of dissolved nickel and copper on the adult coral *Acropora muricata* and its microbiome. Environmental Pollution, 250:792-806. https://doi.org/10.1016/j.envpol.2019.04.030.
I conducted the toxicity test with assistance from A. Reichelt-Brushett and A. Chariton, and staff at the SeaSim. I conducted chemical analyses, carried out DNA extractions, amplification and analysed data. Work at ANSTO was completed by H. Wong, B. Rowling and R Seigler.

## 6.1. Introduction

Coral reefs are increasingly exposed to a variety of anthropogenic stressors. While the effects of climate change (e.g. increase in temperature and decrease in pH) and agricultural run-off (i.e. increasing loadings of pesticides and nutrients) are well documented (Bessell-Browne et al., 2017; Biscere et al., 2015; Flores et al., 2012; Negri et al., 2011; Nystrom et al., 2001), in some environments, metals may also be contributing to the decline in the health of coral ecosystems (Mitchelmore et al., 2007). Exposure to elevated metal concentrations has been shown to result in a range of ecotoxicological effects on corals across all life stages, including coral fertilisation (as presented in Chapter 5), larvae survival and motility (Reichelt-Brushett and Harrison, 2004);,settlement and metamorphosis of larvae (Negri and Heyward, 2001; Reichelt-Brushett and Harrison, 2000), and bleaching (expulsion of zooxanthellae) and photosynthetic efficiency in adult corals (Jones, 1997). Copper is generally more toxic than nickel to all coral life stages (Chapter 5), however, there are far fewer studies that have investigated nickel toxicity to corals. Copper inhibits coral fertilisation, larval metamorphosis and survival between 15 – 150 µg Cu/L (Chapter 5). Survival of adult corals has been shown to reduce by 50% at 250 µg Cu/L (Hedouin et al., 2016b). Nickel has been found to inhibit fertilisation success at exposures >1000 µg Ni/L (Gissi et al., 2017; Reichelt-Brushett and Hudspith, 2016; Reichelt-Brushett and Harrison, 2005) and to inhibit larval survival and settlement at 9000 µg Ni/L (Goh, 1991). To date, there are no reports on the toxicity of nickel to adult corals.

The coral microbiome consists of bacteria, dinoflagellate algae of the genus *Symbiodinium*, viruses, fungi and archaea (Peixoto et al., 2017). The microbiome plays a fundamental role in the development, health and defence of the coral host (Hernandez-Agreda et al., 2017). Microbial communities of the microbiome contribute to carbon and sulfur cycling, phosphorus fixation, metal homeostasis, organic remediation, production of antibiotics and secondary metabolism (reviewed by McDevitt-Irwin et al., 2017). To gain a better understanding of the effects of contaminant exposure on the host, it is desirable to examine corals and their microbiota collectively as ‘holobionts’ (McDevitt-Irwin et al., 2017). Studies on the impacts of climate-change

drivers (sea surface temperature and ocean acidification) have found that environmental stressors can alter microbiome community structure, potentially reducing the health and survival of corals (Grottoli et al., 2018; Thurber et al., 2009; Webster et al., 2016). A number of field studies have shown that the coral microbiome is also susceptible to anthropogenic impacts from sedimentation, sewage and municipal wastewater discharge and changes in salinity (Paulino et al., 2016; Rothig et al., 2016; Zhang et al., 2015; Ziegler et al., 2016).

No studies could be found which have investigated the effects of individual metals on the entire coral holobiont. One study has quantified the impact of copper exposure on tropical marine sponges and their microbiome and showed that bacterial diversity was reduced by 64% following a 48-h exposure to 223 µg Cu/L (Webster et al., 2001). Microbes are key to the functioning and stability of coral reefs, and respond rapidly to environmental change, including declining water quality. Therefore microbes could potentially be used as early warning indicators for environmental stress and coral reef health, rather than traditional monitoring methods based on visual signs of health deterioration (e.g. bleaching) (Glasl et al., 2017). However, there are still significant knowledge gaps concerning the roles of the microbiome and cnidarian host during stress, particularly when exposed to metals.

The aim of this chapter was to investigate how increasing concentrations of dissolved copper and nickel, individually, affects the condition of adult corals and their associated microbiomes, specifically the component associated with the outer surface of the coral host. It was hypothesised that exposures to metal would alter the structure of the coral microbiome, and this could potentially reduce the adaptive ability of corals to deal with stress. The widespread staghorn coral, *Acropora muricata,* was exposed separately to increasing concentrations of copper and nickel, and after 4 days the following endpoints were measured: bleaching (loss of zooxanthellae), accumulation and distribution of metals in corals, and changes in the coral microbiome (using DNA metabarcoding).

## 6.2. Methods

### 6.2.1. General laboratory techniques and reagents

All glassware and plastic containers used in the tests were prepared as described in Chapter 3 Section 3.2.1 and Chapter 5, Section 5.2.1.

All metal stock solutions were prepared volumetrically using high purity water (Chapter 3, Section 32.1.). A copper stock solution of 0.1 g Cu/L and a nickel stock of 1 g Ni/L were prepared.

Physico-chemical parameters (pH, dissolved oxygen (DO), conductivity, salinity and DOC) were measured in the treatment tanks and in one randomly selected replicate chamber every day during the exposure. Parameters were measured using a Multi probe (HQ40d Multi-Hach), calibrated following instructions from the manufacturer.

### 6.2.2. Species collection and maintenance

This toxicity study was carried out at the National Sea Simulator (SeaSim), Australian Institute of Marine Science (AIMS), Townsville, Australia. The scleractinian branching coral, *A. muricata*, was collected by SeaSim on the 7$^{th}$ June 2016 from Trunk Reef (18° 18.173'S, 146° 52.153'E), at 3 – 5 m depth, Great Barrier Reef, Queensland, Australia (GBRMPA Permit number G12/35236.1). One colony of *A. muricata* was separated into 5-8 cm fragments on board the boat and mounted onto aragonite plugs, using super glue (XTRA Loctite super glue, Loctite Australia Pty Ltd). Coral fragments were maintained in 60-L aquaria with flowing seawater (5 L/min) from the collection point, until returned to the SeaSim aquaria on the following day. Once in the SeaSim aquaria, coral fragments were maintained in natural filtered (0.04 µm) seawater, with natural day: night cycles, set to mimic conditions on the reef (2 h ramp up, 8 h at 100 – 150 µmol/m$^2$/s$^1$, 2 h ramp down). Corals were fed a combination of newly hatched *Artemia nauplii* and microalgae (a mixture of *T.Isochrysis lutea, Pavlova lutheri, Dunaliella* sp., *Nannochloropsis oceania, Chaetoceros muelleri*, and *Chaetoceros calciltrans* at 5 x 10$^6$ cells/mL).

### 6.2.3. Toxicity testing with adult corals

Toxicity tests commenced on the 15th July 2016, approximately 5 weeks after acclimation to aquaria conditions. There is no established protocol for acclimating adult corals prior to testing in aquaria conditions; however, the health of the corals was noted by observing the colour of the fragments and the presence of skeletal growth around the base of the fragments on the aragonite plugs.

Treatment solutions were made in 80 L Perspex tanks by diluting metal stock solutions in natural filtered (0.04 µm) seawater. At the SeaSim, incoming seawater passes through a hydro cyclone, arkal disk filters (100 µm), followed by a foam fractionator and finally a memcor ultrafiltration system (0.04 µm). Filtered seawater was stored in a covered dam before delivery to the experimental systems. On Day 0, 40 L of each treatment solution was prepared. To top up the tanks on subsequent days (Days 1-3), 20 L of each treatment solution was prepared and added to the respective tanks. Treatment solutions were fed to test chambers via linear low-density polyethylene (LLDPE) tubing, using peristaltic pumps (Masterflex® L/S Digital Std drive. Extech Equipment Pty Ltd, Vic, Australia). Test chambers were 2.5 L, with clear acrylic lids (to allow for light penetration) and a PVC container. Treatment solutions were delivered into the top of each chamber, with submersible magnetic stirrers to provide water movement (Figure 6.1). Test chambers were housed in a single water bath to permit accurate temperature control. The total volume of treatment solution in each chamber was 2 L and the flow rate for each chamber was 2.8 mL/minute, which resulted in an 80-90% water exchange, twice every 24 h. Coral fragments on aragonite plugs were inserted into the mounting plates in each chamber (Figure 6.1). There were four replicate chambers per treatment, with three fragments in each chamber. Full details on the toxicity test parameters are provided in Table 6.1.

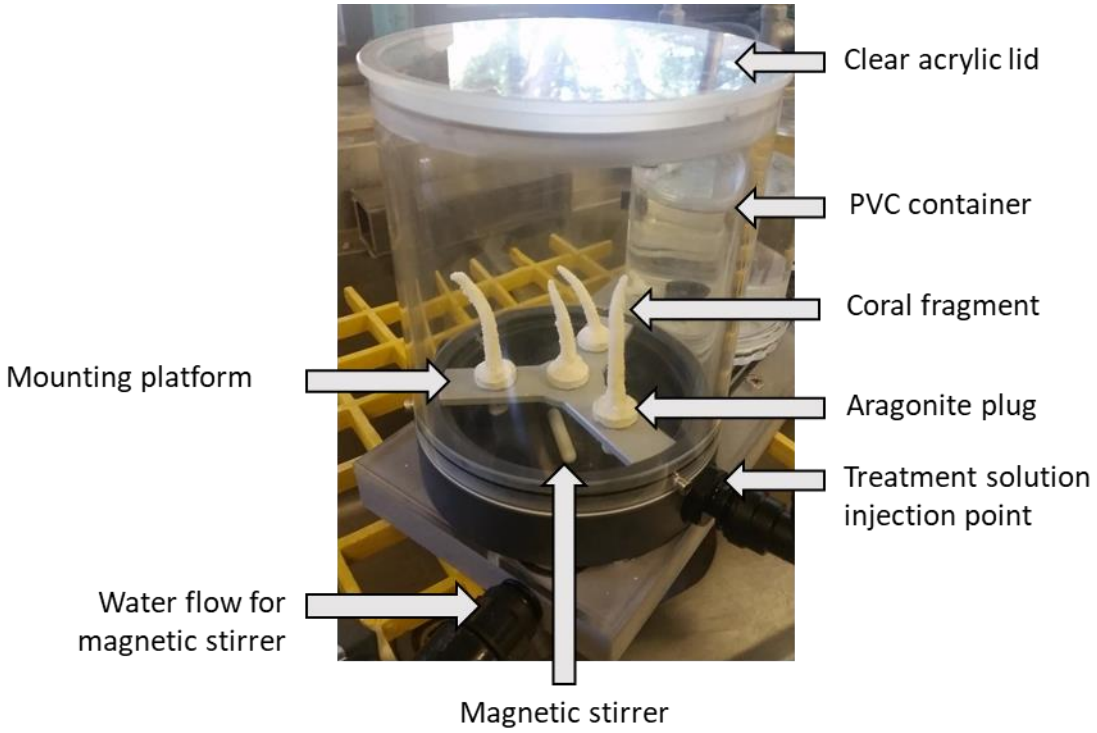



Figure 6.1. Design of the coral test chamber. Note that only 3 coral fragments were placed in each chamber.

At 36 h, severe bleaching was observed in the following treatments: 20, 50 and 100 µg Cu/L and 10 000 µg Ni/L (nominal values). To ensure that these treatments could still be sampled for tissues, these fragments were sampled and processed (described below) at 36 h. On Day 4, remaining fragments were removed from the chambers, rinsed in seawater (note: fragments collected at 36 h were not rinsed in seawater due to severe tissue degradation), and photographed for assessment of bleaching. The coral watch health chart was used to assess the degree of bleaching in coral fragments following exposure (Coral Watch, 2014). Three fragments from each chamber were processed for the following measurements:

(i) Fragment 1: Quantification of metals in coral tissues. The fragment was rinsed in clean seawater, placed in a clean/new ziplock bag and air blasted to remove tissues which were then transferred into a 5 mL polypropylene vial and acidified to 2% $HNO_3$ (Tracepur, Merck).

(ii) Fragment 2: Spatial distribution of metals in coral fragments. The fragment was rinsed in clean seawater put in a clean/new ziplock bag, made air tight and stored in a freezer (-20$^{o}$C).

(iii) Fragment 3: Changes in coral microbiome community structure using DNA metabarcoding. The fragment was rinsed in seawater, placed in clean/new ziplock bag and air blasted to remove external tissues. The tissue slurry was pipetted (using sterile pipettes) into 2 mL cryo vials and flash frozen in liquid nitrogen and stored at -80°C until DNA extraction.

### 6.2.4. Quantification of metals in coral tissues

Nickel and copper-exposed coral tissue samples were transferred to pre-weighed, acid-washed, 50 mL digest tubes. Samples were oven dried at 60°C for 24 h and weighed to determine the dry mass (0.08 ± 0.03 g) of sample to be digested. Dried tissues were combined with 5 mL of concentrated $HNO_3$ (Tracepur, Merck) and left with loosened lids in a fume hood overnight. Tubes were then heated (hot block, Digi Prep MS, SCP Science) to 60°C for 2 h (with a 30 min ramp up to the max temperature). Digested samples were allowed to cool in a fume hood and diluted to a final concentration of 6.6% $HNO_3$ with high purity water. Samples were analysed by ICP-AES (Chapter 3, Section 3.2.4). Samples included tissues, three laboratory blanks (seawater, from the time of sampling the corals), three digestion blanks, and three certified reference material samples (National Institute of Standards and Technology (NIST), Standard Reference Material, SRM 2976) matching the weight range (dry weight, 0.08 ± 0.03 g) of the coral samples.

Table 6.1. Toxicity test conditions and parameters for 96-h exposure with *Acropora muricata*

| **Test conditions/parameters** | |
|---|---|
| Temperature (°C) | 27 ± 0.5 |
| Salinity (‰) | 35 ± 1 |
| DO (%) | >80 |
| pH | 8.1 ± 0.2 |
| Nickel treatments (µg/L) | 50, 100, 500, 1000, 10000 |
| Copper treatments (µg/L) | 5, 20, 50, 100 |
| Replicates | 4 replicate chambers per treatment |
| Light parameters | Low light 6:30 (dawn)<br>Full light 8:00 (100-150 µmol/$m^2$/s). Irradiance was similar to the expected mean photosynthetically active radiation at the collection site.<br>Low light 16:00 (dusk)<br>Lights off 18:00 |
| Test type | Flow through: 2.8 mL/day/chamber, 80-90% of water exchanged 2 x per day in each chamber. |
| Test chamber | 2.5 L polycarbonate container and lid, water circulation within chamber maintained with magnetic stirrer bar controlled by water pressure (Figure 6.1) |
| Test volume | 2 L |
| Test duration | 96 h |
| Control/diluent water | 0.04 µm filtered natural seawater |
| Life stage of test organism | Adult, 5-8 cm fragments |

### 6.2.5. Spatial distribution of nickel in coral fragments

To investigate the distribution of nickel in the coral fragments, selected coral samples were analysed using ITRAX x-ray fluorescence, laser ablation ICPMS (LA-ICPMS) and micro particle-induced x-ray emission spectrometry (µ-PIXE) at ANSTO, NSW, Australia. For the ITRAX analysis, one control and one nickel-exposed fragment (500 µg Ni/L) were analysed. No significant difference between the control and nickel-exposed coral fragment was detected and so this method was not pursued further (data not shown).

For the LA-ICPMS technique, one control coral fragment was analysed alongside one nickel - exposed fragment from each of the following treatments, 100, 1000 and 10 000 µg Ni/L (nominal). Prior to analysis, coral fragments were half embedded longitudinally in paraffin wax and cut into four (11 mm) sections (termed A-D) using a diamond wire (Appendix E, Figure E2). Sections B, C and D of the coral fragment exposed to 10 000 µg Ni/L were analysed first, while subsequent measurements of other coral fragments were taken from Section B only. Prior to analysis, sections were polished/sanded by hand using a 220-grit size SiC paper, to level the

surface of the coral and wax, and then cleaned with high purity water and air dried (at room temperature). Samples were analysed using a Resonetics M50 193nm Excimer laser ablation system coupled to a Varian-820 –ICPMS. A rectangular laser spot (20 µm x 100 µm) and a laser pulse frequency of 10 Hz was used. Ablation paths were cleaned by laser at a rate of 150 µm/s prior to analysis at 30 µm/s with helium and nitrogen flow rate of 600 and 5 mL/min, respectively, through the sample cell. Mass spectrometry was conducted with a dwell time of 20 ms. All elements were referenced to NIST SRM612 (trace elements in glass). NIST glass references are not certified for magnesium, however a reference value supplied by the Iolite software (77 µg/g) was used (Runnalls and Coleman, 2003). Mass spectrometry data were processed with Iolite. It was not possible to normalise data using $^{43}Ca$, as is standard practice (Limbeck et al., 2015), due to the highly porous nature of the coral samples. Therefore, data presented for nickel exposures are semi-quantitative only.

To support the LA-ICPMS data, Sections B and D of the coral exposed to 10 000 µg Ni/L (nominal) were analysed with micro Particle-Induced X-ray Emission (µ-PIXE) using the Australian National Tandem Research Accelerator heavy ion microprobe (Siegele et al., 1999) with a 3-MeV proton beam with a spot size of approximately 5-7 µm and a beam current of 0.3 - 1.0 nA. X-ray fluorescence spectra were collected using a high-purity Ge detector with an active area of 100 $mm^2$ approximately located 33 mm from the sample. To reduce low-energy x-rays and to prevent scattered protons from entering the detector, a 112 µm thick Mylar foil was placed in front of the detector. Samples were scanned over an area of approximately 2×2 mm, which is the maximum scan area for 3-MeV protons achievable with the ANSTO microprobe. The µ-PIXE data were analysed using GeoPIXE software (Ryan, 2001; Ryan et al., 1995) and elemental maps were extracted from the data.

### 6.2.6. Chemical analyses

Water samples were taken from the filtered seawater entering the aquaria (used to make treatment solutions) and the treatment tanks every day during the 96-h exposure. On Day 0 and at the time coral fragments were removed from chambers (36- and 96-h), sub-samples were

taken from all test chambers. Samples were analysed using ICP-AES (Chapter 3, Section 3.2.4). For tissue digests, values were reported as metal concentrations in µg/kg dry weight.

Sub-samples were taken from the seawater used to prepare the treatment solutions and from one replicate chamber at test completion to measure DOC (Chapter 3, Section 3.2.4). Samples were taken on Day 0 and at test completion.

### 6.2.7. DNA extraction amplification and sequencing

Samples were removed from the -80°C freezer, gently thawed and extracted using the QIAGEN® DNeasy Power Biofilm kit (QIAGEN®, Germany), according to manufacturer's instructions with the following modifications; the Fast Prep®-24 (MT™) was used to lyse the samples for 45 seconds, with the speed set to 4.5; DNA was eluted in 2x 50µL of elution buffer and allowed to rest for 1 min before the final centrifugation step. Success of extraction and DNA yield was measured on the Nanodrop spectrophotometer (Thermo Fisher Scientific, USA).

Three different sets of primers were used to target and amplify different components of the microbiotic community in the coral tissues. The eukaryotic community was determined using the All18SF (5'-3': TGGTGCATGGCCGTTCTTAGT) and All18SR (5'-3': CATCTAAGGGCATCACAGACC) primers for the V7 region of the 18S rRNA gene (Hardy et al., 2010). The bacterial composition was determined with two different primer sets to identify the most appropriate primer set. The first 16S primer set was 515f (5'-3': GTGYCAGCMGCCGCGGTAA) (Baker et al., 2003; Quince et al., 2011), 806r (5'-3': GGACTACNVGGGTWTCTAAT) (Apprill et al., 2015) for the V4 region of the 16S rRNA gene (recommended by Earth Microbiome Project, EMP). The second primer set was 784f (5'-3': AGGATTAGATACCCTGGTA), 1061r (5'-3': CRRCACGAGCTGACGAC) for the V5 and V6 region of the 16S rRNA gene (Andersson et al., 2008; Ziegler et al., 2016). Results showed that the overall analysis of the community composition using either the V4 primers or the V5-V6 primers were similar; and the V4 primers provided a better coverage of the bacterial community. For these reasons only results for the V4 primers are presented here; results for V5-V6 primers are provided in Appendix E.

The conditions for the polymerase chain reaction (PCR) used to amplify the rDNA with each of the primer sets is described in Appendix E, Table E1. All amplifications used the Amplitaq Gold 360 Master Mix (MM, Applied Biosystems) and DNA-free water (Millipore®). For 18S rDNA, the total PCR reaction volume was 50 µL which consisted of 25 µL of MM, 1 µL of each primer (10 µM), 20 µL of water and 3 µL of template DNA. For 16S rDNA, PCR reactions followed methods available on the EMP website, and the total reaction volume was 25 µL, with 10 µL of MM, 0.5 µL of each primer (10 µM), 11 µL of water and 3 µL of template DNA. In all PCR reactions, samples were amplified alongside positive controls (mussel or crocodile DNA for 18S rDNA; Enterobacter for the 16S rDNA and negative controls (DNA-free water)). Primers were barcoded for multiplexing with each sample given a unique barcode combination following the protocol of Chariton et al. (2015).

Following amplification of either 18S rDNA or 16S rDNA, samples were pooled (separately) and PCR products were purified using the QIAGEN QIAquick® PCR purification kit. Amplification and purification success was interrogated on a MultiNA gel (Shimadzu, MCE-202), following the manufacturer's instructions. Negative controls were checked for contamination in the MultiNA gel (no contamination was detected in any PCRs used for sequencing) and were not included in the pooled samples processed for sequencing. The final pooled amplicon library concentrations were measured on the Nanodrop and sent to the Ramaciotti Centre for Genomics (University of New South Wales, Australia) for sequencing. Amplicon libraries were prepared for sequencing using TruSeq PCR-free kit. As the base pair sizes of the amplicons were different for 18SrDNA (180 bp) and 16SrDNA (350 bp), the libraries were run as two separate Illumina® Miseq sequencing runs, 2x 250 bp for 16S and 2x 150 bp for 18S. Raw sequences are available at https://doi.org/10.25919/5b9745a02208a.

### 6.2.8. Bioinformatics

Sequenced data were processed using a custom pipeline (Greenfield Hybrid Amplicon Pipeline, GHAP) which is based on USearch tools (Edgar, 2013). The pipeline is available at https://doi.org/10.4225/08/59f98560eba25. GHAP demultiplexes the sequence reads to produce

a pair of files for each sample. These paired reads were merged, trimmed, de-replicated, and clustered at 97% similarity to generate a set of representative OTU (Operational Taxonomic Units) sequences. USearch v10.0.240 tools (fastq_mergepairs, fastx_uniques and cluster_otus) (Edgar, 2013) were used for the merging, de-replicating and clustering steps. The 16S rDNA OTU sequences were classified in two ways: (i) by using the RDP Classifier (v2.12) (Cole et al., 2014) to determine a taxonomic classification for each sequence, down to the level of genus where possible; and (ii) by using usearch_global to match the representative sequence from each OTU against a 16S rRNA reference set built by merging the curated sequences from the RDP 16S training set (release 16) and the RefSeq 16S rRNA set (downloaded in July 2017). The 18S rRNA representative sequences were classified by matching them (ublast) against a curated set of 18S reference sequences derived from the SILVA v123 SSU reference set (Cole et al., 2014; Quast et al., 2012). This 18S rDNA reference set was built by taking all the eukaryote sequences from the SILVA v123 SSU dataset, and removing those sequences containing bacterial or chloroplast regions, as well as those with inconsistencies in their taxonomic lineages. A full description of this curation is provided in the GHAP documentation. The pipeline then used usearch_global to map the merged reads from each sample back onto the OTU sequences to obtain accurate read counts for each OTU/sample pairing. The classified OTUs and the counts for each sample were finally used to generate OTU tables in both text and BIOM (v1) file formats, complete with taxonomic classifications, species assignments and counts for each sample. Operational taxonomic unit (OUT) is an operational definition that is used to classify groups of closely related organisms based on similar marker gene sequences (Scholz, 2018).

### 6.2.9. Statistical analyses

After processing through the bioinformatics pipeline and prior to statistical analysis, data were processed through a final filtering step. For 18S and 16S data, the highest reads for the positive control OTU in samples were 60 and 167, respectively. These two values were used as the cut-off points for filtering the dataset, with 18S rDNA and 16S rDNA OTUs with maximum detections of 60 or 167 reads, respectively, deleted, thereby removing potential tag-jumped sequences and

low-quality reads. OTUs which had a match percent of <80 were also removed. The positive controls amplified in the PCR were also used for screening of successful amplification and sequencing and to check for cross-contamination in the 18S and 16S libraries. The positive controls and OTUs were removed from the dataset and this final dataset was used in the statistical analysis. For the 18S rDNA data, all coral OTUs were also removed to focus on the microbiota only. Appendix E, Table E2 shows the total number of reads and OTUs prior to and following this filtering step.

The data were not rarefied because of no or a weak correlation between number of sequence reads and organism abundance (18S rDNA $R^2$ = -0.002, 16S rDNA $R^2$ = 0.3) (Egge et al., 2013). The 18S rDNA dataset was transformed to presence/absence prior to computation (Chariton et al., 2015). The 16S rDNA data were initially standardised by the total abundance and then square-root transformed to calculate the relative abundance of each OTU across samples.

Multivariate analysis of the microbiome data was performed using the Primer 7+ statistical package (Plymouth Marine Laboratory, UK). Ordination was performed by non-metric multidimensional scaling (nMDS) using the Bray-Curtis similarity coefficient. Statistical differences between treatments were tested by permutational multivariate analysis of variance (PERMANOVA, $P \leq 0.05$), based on 999 random permutations. Primer's SIMPER function was used to identify key taxa contributing to compositional differences between treatments, using Bray-Curtis similarity, one-way design and the cut-off percentage set to 90. For the 18S rDNA and both 16S rDNA datasets, the taxonomic levels of class and family, respectively were used. Shade plots to indicate number of OTUs in each sample were also generated in Primer.

The DIVERSE function in Primer was used to determine Shannon's diversity. Differences in these univariate attributes across treatments were examined using a one-way ANOVA with Bonferroni's (all pairs) and Tukey-Kramer tests in NCSS v7 (Utah, USA).

## 6.3. Results

### 6.3.1. Quality control

After 36 h, fragments exposed to nominal concentrations of 20, 50 and 100 µg Cu/L and 10,000 µg Ni/L had to be terminated and coral fragments retrieved because bleaching had commenced. Fragments in the remaining treatments (control, and nominal copper concentrations of 5 µg Cu/L, and nominal nickel concentrations of 50, 100, 500 and 1000 µg Ni/L were exposed for 96 h.

Over the 96-h exposure period, physico-chemical parameters were maintained within acceptable limits (Table 6.1). Dissolved organic carbon in the filtered aquaria seawater was 1.7 mg/L on Day 0. In treatment chambers sacrificed at 36 h, DOC was 1.9 – 4.3 mg/L; at 96 h, DOC in the remaining chambers 1.7 – 2.2 mg/L. Variable DOC concentrations measured at 36 h is reflective of the degree of tissue degradation of the unhealthy corals in these treatments.

The background concentrations of metals in the seawater used to make treatment solutions were generally below the LOD (Appendix E, Table E3). The mean of the measured dissolved concentrations in the chambers on Days 0 and 4 (or at 36 h for some treatments) was used in all following analyses (Table 6.2). Throughout the exposure the proportion of metal in the dissolved phase in the test chambers was 98-100% for nickel treatments and 75-98% for copper treatments. There was minimal decline in dissolved nickel concentrations (<1%) over 96 h, likely due to daily renewal of test solutions. Dissolved copper concentration decreased between 10-58% over the exposure period. The greatest loss in dissolved copper was observed in the lowest nominal copper treatment of 5 µg/L and this is most likely a reflection of uptake or adsorption by coral tissues and algal endosymbionts. The measured, total and dissolved concentration of nickel and copper in the tanks and chambers are provided in Appendix E, Tables E4 and E5.

Table 6.2. Concentrations of dissolved nickel and copper, measured in the test chambers on day 0 (initial) and at 36 or 96 h. Reported values are the mean and standard deviation (SD, n=4).

| | Initial | | 36 or 96 h[a] | | |
|---|---|---|---|---|---|
| **Treatment** | **Mean** | **SD** | **Mean** | **SD** | **Mean of Initial and 36 or 96 h** |
| **Nominal (µg/L)** | **Measured dissolved metal (<0.45 µm) (µg/L)** | | | | |
| | | | Nickel | | |
| Control[b] | 0.9 | 0.6 | 0.9 | 0.3 | 0.9 |
| 50 | 43 | 0.6 | 46 | 0.5 | 45 |
| 100 | 87 | 0.6 | 93 | 0.5 | 90 |
| 500 | 467 | 7.9 | 477 | 2.5 | 470 |
| 1000 | 873 | 8.0 | 922 | 3.9 | 900 |
| 10000[c] | 8910 | 136 | 9190 | 38 | 9050 |
| | | | Copper | | |
| Control | 3.2 | 0.5 | 0.8 | 0.2 | |
| 5 | 5.5 | 0.2 | 2.3 | 0.1 | 4 |
| 20[c] | 14 | 0.7 | 9.2 | 0.7 | 11 |
| 50[c] | 35 | 1.1 | 29 | 0.4 | 32 |
| 100[c] | 68 | 2.1 | 61 | 2.2 | 65 |

[a] For corals that were removed at 36 h, metals were sub-sampled and for these treatments the mean dissolved concentration sampled at 36 h was used. For all other treatments where the exposure ran for 96 h, the mean dissolved concentration sampled at 96 h was used.
[b] Where concentrations were below the LOD, values were supplemented with half LOD
[c] These treatments were sacrificed and samples taken for analysis at 36 h (not 96 h), due to bleaching.

### 6.3.2. Response of coral to exposure to nickel and copper

Full colour and no bleaching was observed in the control (unexposed) coral fragments, indicating 100% survival following 96-h exposure (Figure 6.2). The lowest tested copper and nickel concentrations of 4 µg Cu/L and 45 µg Ni/L did not cause coral bleaching over 96 h. After 36-h exposure, bleaching was observed in copper treatments of 11, 32 and 65 µg Cu/L and the highest nickel treatment of 9050 µg Ni/L. After 96-h exposure bleaching was observed in nickel treatments of 470 and 900 µg Ni/L. Coral fragments from each treatment were given scores according to the Coral Watch health chart (Appendix E, Table E6).

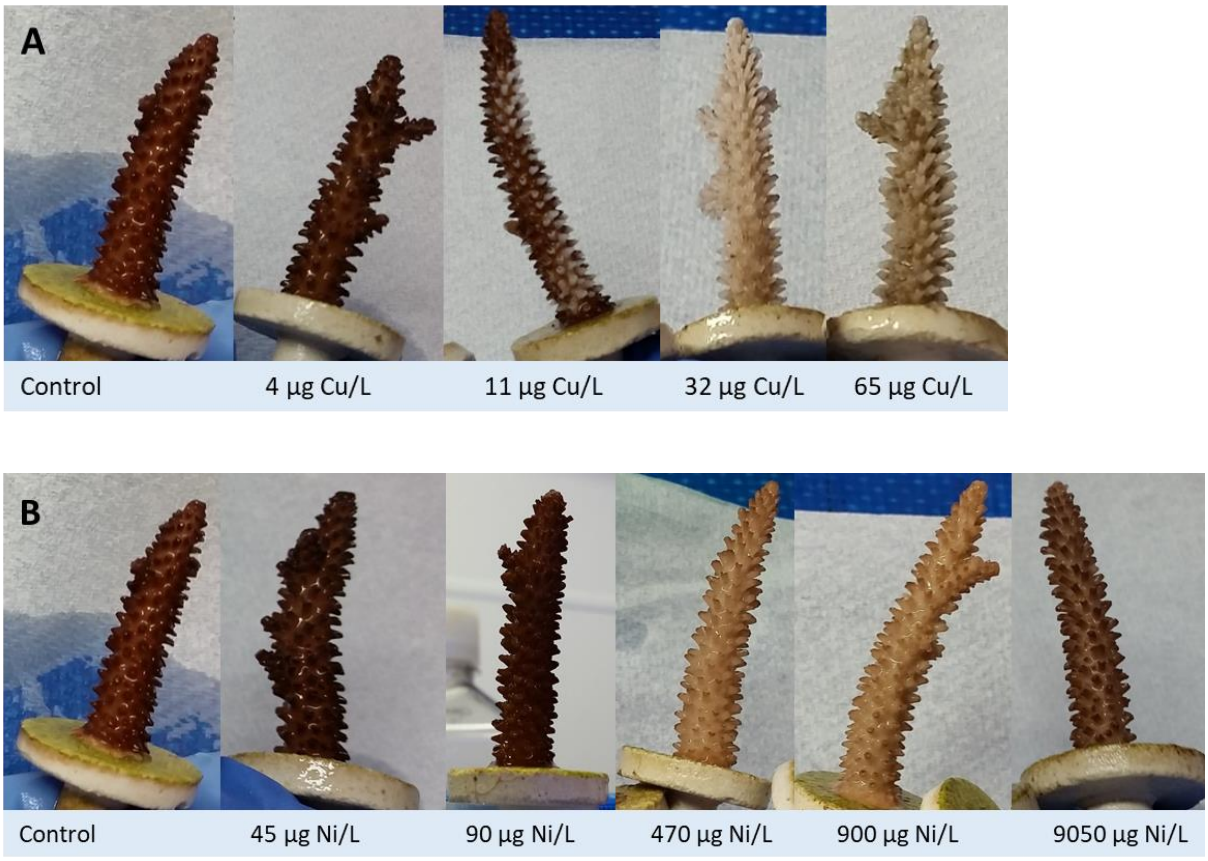



Figure 6.2. Photographs of the coral fragments following exposure to copper (A) and nickel (B) for 36-96 h. For 11, 32 and 65 µg Cu/L and to 9050 µg Ni/L, corals were exposed for 36 h, with all remaining treatment exposures for 96 h. Photographs were taken as soon as corals were removed from the test chambers. Each photograph is one representative replicate per treatment. Treatment concentrations are the measured, dissolved values (Table 4).

### 6.3.3. Metal uptake and distribution in corals

Concentrations of nickel and copper in coral tissues increased with increasing exposure concentration (Figure 6.3). The metal tissue concentration per surface area showed a similar trend (Appendix E, Figure E1).

### 6.3.4. Spatial distribution of nickel in coral fragments

Analysis by LA-ICPMS showed that nickel accumulated in a higher proportion in the proximal end (Section B) than in the distal sections (C, D) of the coral fragment exposed to 9050 µg Ni/L (Appendix E, Figure E3). For Section B of each treatment, there was a peak in nickel concentration detected around the polyps of the coral fragments. The relative proportion of nickel in the coral fragments increased with increasing exposure concentration (Appendix E, Figure E3, E4). This is correlated with a decrease in calcium detection, indicative of organic tissues. The LA-ICPMS data were in agreement with elemental maps generated using µ-PIXE which also detected peaks in nickel around the polyps that correlated with a decrease in calcium. The µ-PIXE data also showed a peak in nickel within the axial (central) polyp (Appendix E, Figure E5, E6).

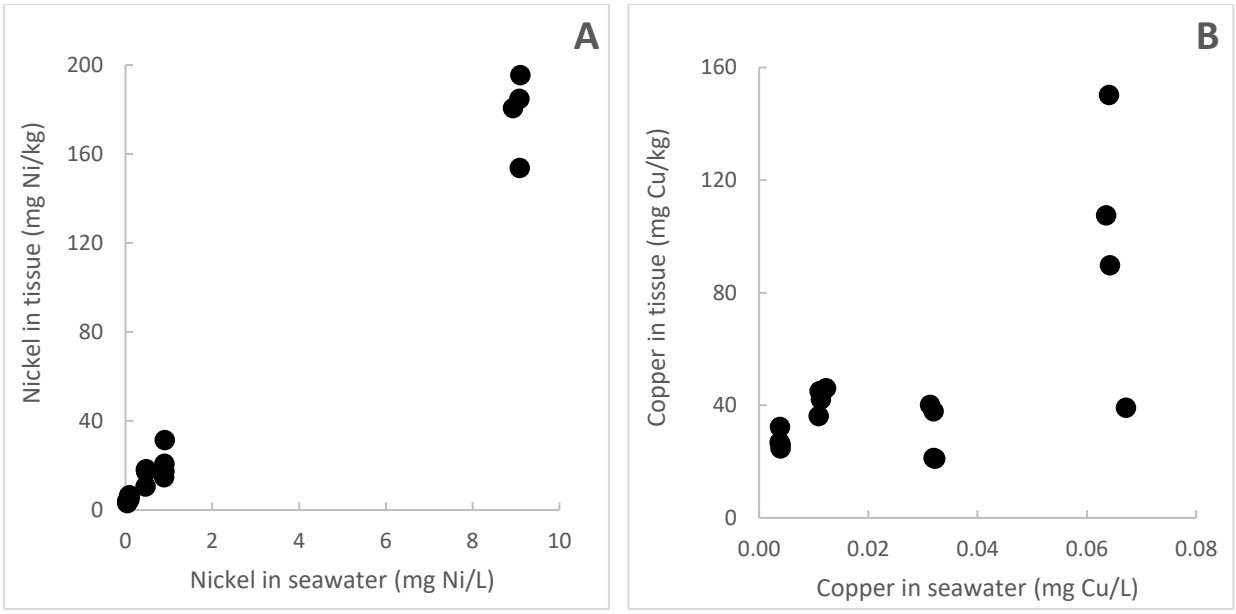



Figure 6.3. The effect of measured, dissolved (0.45 µm) metals in seawater on coral tissue concentrations for nickel (A) and copper (B). Each point represents one individual fragment from four replicate chambers per treatment. Note the different scales on the x and y axes.

### 6.3.5. Community structure of the coral microbiome

All 18S data were transformed to presence/absence, therefore the analysis described below is referring to differences in richness of the OTUs associated with each taxonomic group. All 16S data were square root transformed, therefore analysis of 16S data refer to the relative abundances of the taxonomic groups across treatments. DNA extraction and amplification of coral tissues exposed to 900 and 9050 µg Ni/L were unsuccessful, possibly due to insufficient tissue. Consequently, statistical analyses of the DNA metabarcoding results for nickel exposure were restricted to the control, 45, 90 and 470 µg Ni/L treatments.

*Nickel - Eukaryotes*

Following 96-h exposure, the diversity in eukaryote (18S rDNA) taxa in the coral microbiome appeared to decline with increasing nickel concentration (Figure 6.4 A); however, this was not statistically significant (ANOVA $F = 1.06$, $p = 0.22$). This may be due to the large variation in diversity observed at 90 and 470 µg Ni/L and the relatively small sample size. Figure 6.4 B shows a statistically significant change in the composition of the eukaryotic community (PERMANOVA $F = 2.9$, $P = 0.01$), with the composition of the microbiomes from the highest nickel treatment (470

µg Ni/L) being different to the control and the lowest nickel treatment (45 µg Ni/L, $P < 0.05$) (Figure 6.4 B).

The three major taxa that contributed to the differences between the control and highest nickel treatment were OTUs associated with Chromadorea (26% contribution), Chlorophyceae (11%) and Bacillariophyceae (11%) (SIMPER analysis). When comparing the lowest nickel treatment (45 µg Ni/L) to the highest (470 µg Ni/L), the two major taxa that contributed to the differences were OTUs associated with Chromadorea (33%) and Phaeophyceae (10%). As nickel concentration increased there was a decrease in the OTUs associated with Chromadorea and Phaeophyceae, and an increase in Chlorophyceae and Bacillariophyceae (Figure 6.4 C). Dinophyaceae OTUs, which includes *Symbiodinium,* remained unchanged across all treatments.

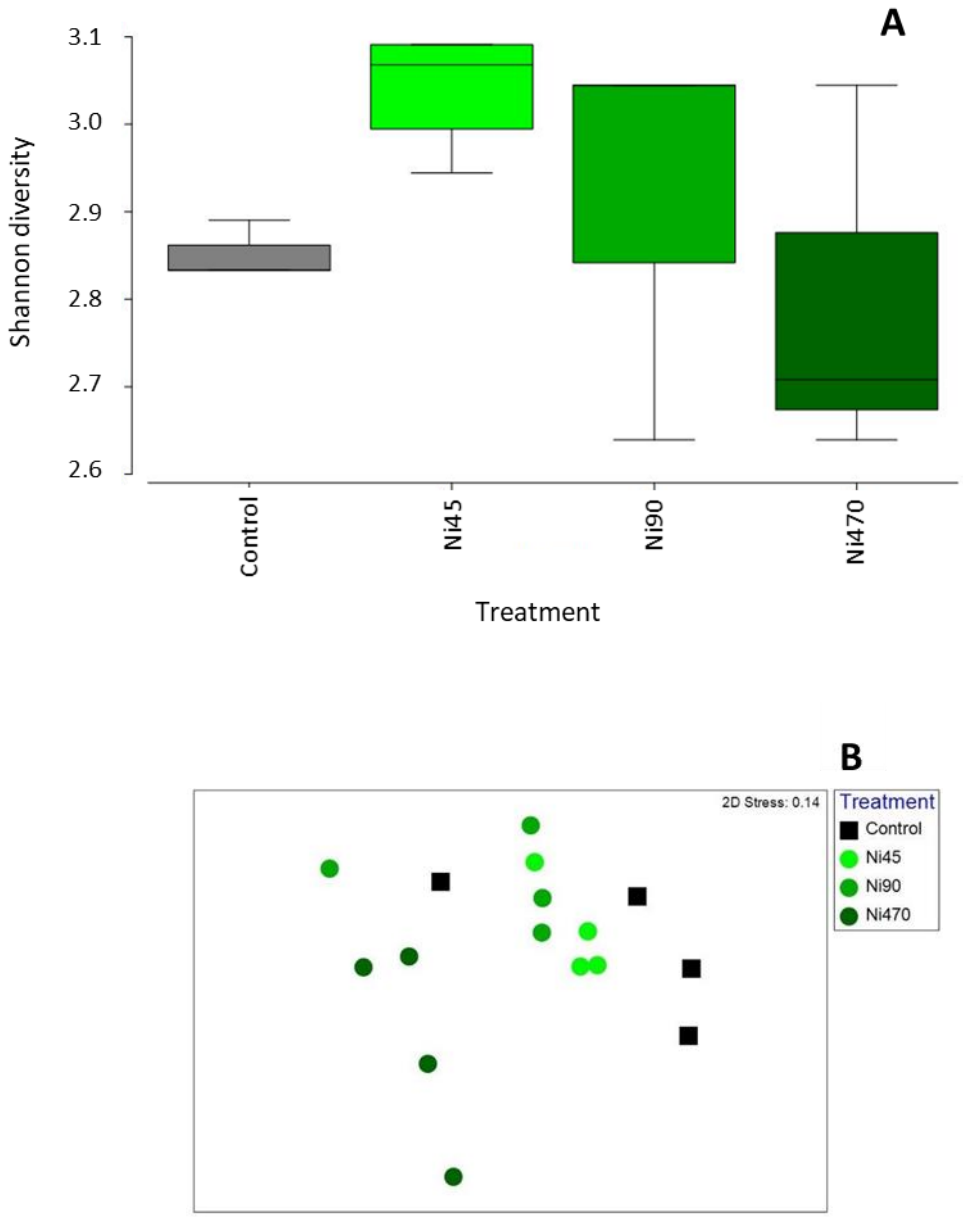



Figure 6.4. The effect of nickel on the eukaryote community composition of the coral microbiome following 96-h exposure A) Boxplots showing the variation in Shannon diversity (median and variation of 4 replicates) across control and nickel treatments B) Non-metric multidimensional scaling plot showing the relative similarity of the 18S community composition. Each point represents one individual replicate from each treatment. C) Shade plot demonstrating the changes in eukaryote taxa in response to increasing concentrations of nickel. Taxonomic level = class, OTU = Operational Taxonomic Units. Ni45 = 45 µg Ni/L and so on. Metal concentrations are measured dissolved values in µg/L.

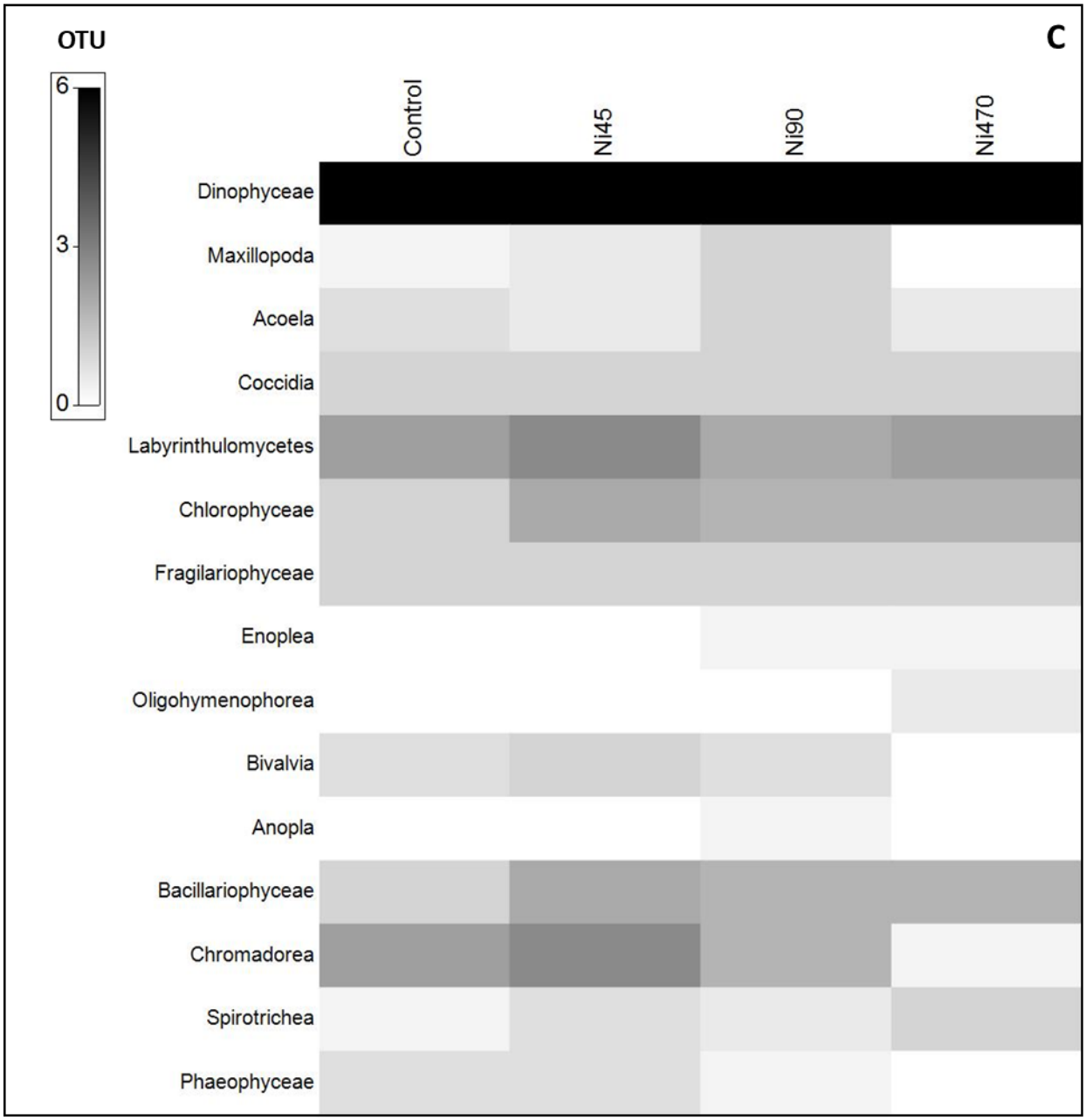



Figure 6.4. Continued.

*Nickel – Bacteria*

There was no effect of nickel exposure on Shannon diversity of the bacterial community of the coral microbiome following exposure to control and nickel treatments (ANOVA F = 0.94, p = 0.45, Figure 6.5 A). Dissolved nickel exposures did not alter bacterial community composition, compared to seawater only (control) (PERMANOVA F = 1.7, P = 0.11), however, there did appear to be a slight separation among the treatments (Figure 6.5 B).

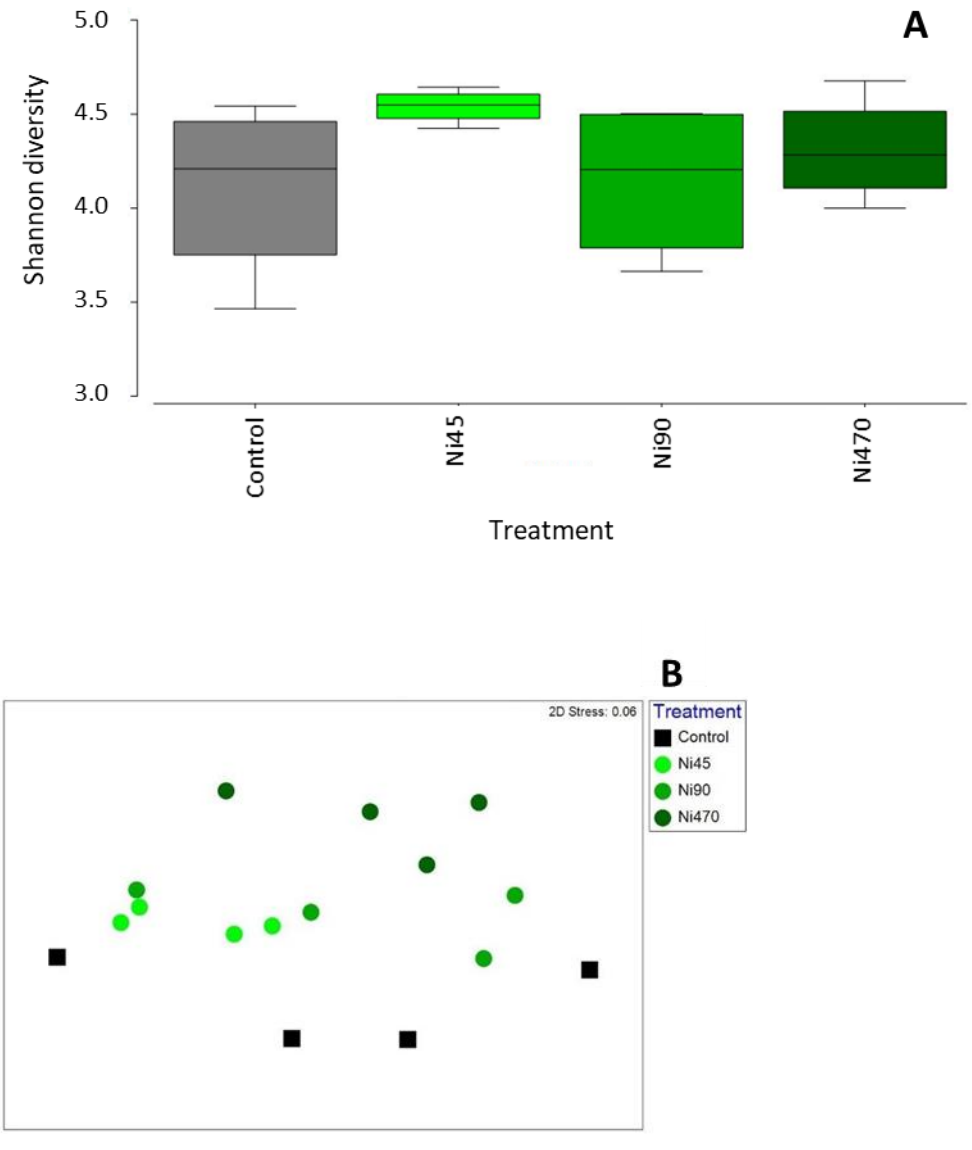



Figure 6.5. The effect of nickel on the bacterial community composition of the coral microbiome following 96-h exposure. A) Boxplots showing the variation in Shannon diversity (mean and variation of 4 replicates) across control and nickel treatments. B) Non-metric multidimensional scaling plot showing the relative similarity of the 16S community composition. Each point represents one individual replicate from each treatment. Ni45 = 45 µg Ni/L and so on. Metal concentrations are measured dissolved values in µg/L.

*Copper - Eukaryotes*

Following 36 – 96-h exposure to copper, there was a decrease in the eukaryote diversity of the coral microbiome with increasing copper concentrations (Figure 6.6 A, ANOVA $F = 5.2$ and $p = 0.007$); 65 µg Cu/L was significantly different to the control and 4 µg Cu/L ($p<0.05$). It should be noted that corals were exposed to copper treatments of 11, 32 and 65 µg Cu/L for 36 h, while control and 4 µg Cu/L were exposed for 96 h.

Copper exposure was shown to alter the eukaryote community structure of the microbiomes of *A. muricata* (PERMANOVA $F = 3.4$, $P = 0.001$) (Figure 6.6 B). The control and the lowest copper treatment (4 µg Cu/L) had similar eukaryote communities ($P > 0.05$), and the two highest copper treatments (32-65 µg Cu/L) had significantly different community structure compare to the control, and 4 and 11 µg Cu/L treatments ($P < 0.05$).

The taxa that were contributing to the differences between the control and the highest copper concentrations (11, 32 and 65 µg/L) were OTUs associated with Chromadorea (23-29%), Labyrinthulomycetes (10-18%), Bacillariophyceae (10-11%) and Chlorophyceae (9-11%). As copper concentration increased, there was a decrease in the OTUs associated with Chromadorea, Labyrinthulomycetes and Bacillariophyceae, with a subsequent increase in OTUs associated with Chlorophyceae (Figure 6.6 C). The number of *Symbiodinium* OTUs, represented by Dinophyceae did not change across all treatments (Figure 6.6 C).

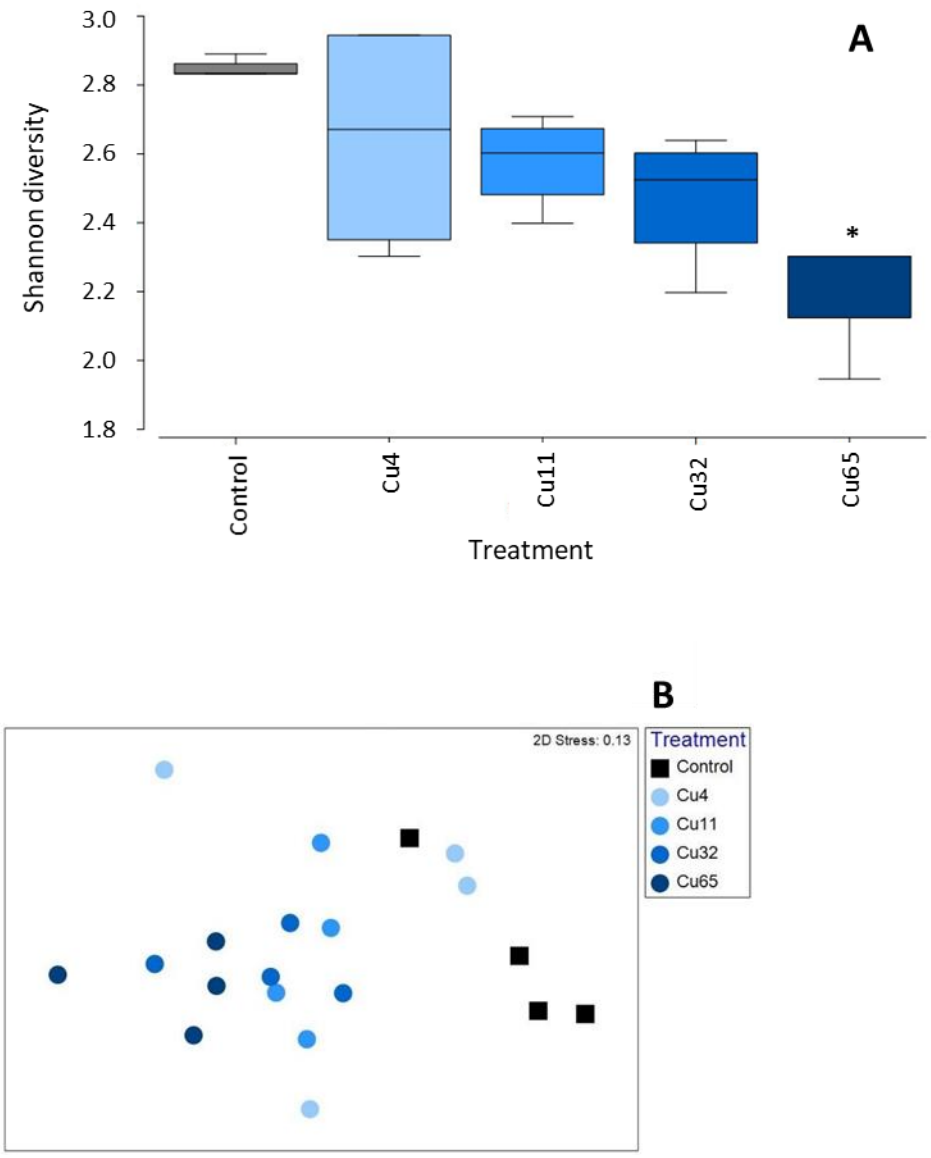



Figure 6.6. The effect of copper on the eukaryote community composition of the coral microbiome following 36 – 9 6-h exposure. A) Boxplots showing the variation in Shannon diversity (mean and variation of 4 replicates) across control and copper treatments. B) Non-metric multidimensional scaling plot showing the relative similarity of the 18S community composition. Each point represents one individual replicate from each treatment. C) Shade plot demonstrating the changes in the presence/absence of eukaryote taxa in response to increasing concentrations of copper. Taxonomic level = Class, OTU = Operational Taxonomic Units. Cu4 = 4 µg Cu/L and so on. Metal concentrations are measured dissolved values in µg/L.

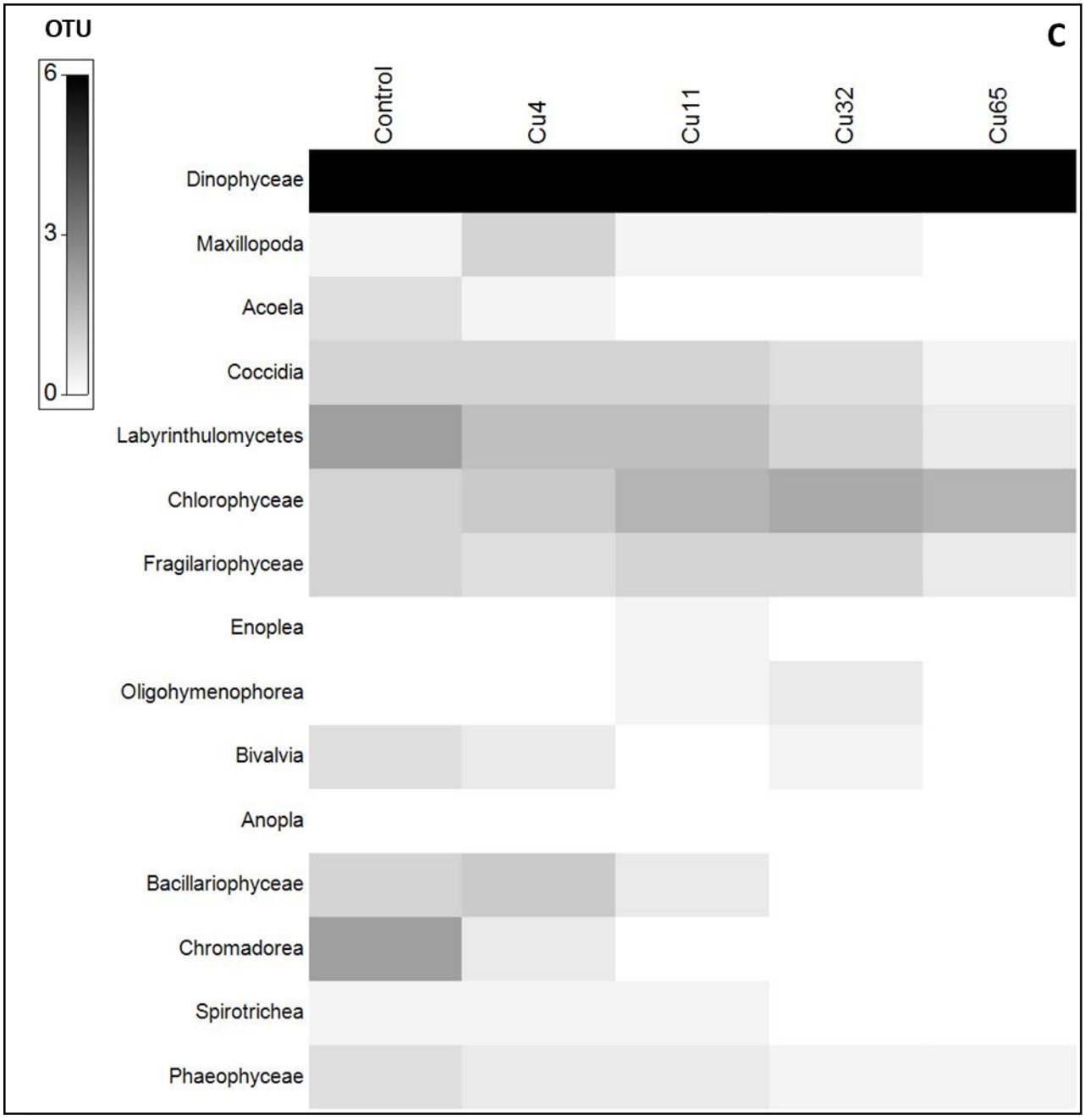



Figure 6.6. Continued.

*Copper – Bacteria*

While the data suggested that copper decreased bacterial diversity (Figure 6.7 A), this was not found to be statistically significant ($p>0.05$). However, variation in diversity was markedly lower in the two highest treatments (Figure 6.7 A).

Similar to the 18S rDNA data, the control and the lowest copper treatment (4 µg Cu/L) had a similar community composition (PERMANOVA $F = 5.7$, $P = 0.001$, Figure 6.7 B), and as copper concentration increased, the structure of the bacterial community changed significantly. The two highest copper treatments (32 and 65 µg Cu/L) were significantly different to the control, and 4 and 11 µg Cu/L treatments (PERMANOVA $P <0.05$).

The significant differences between the copper treatments (11, 32 and 65 µg/L) and the control were driven by Flavobacteriaceae (22-26%) Rhodobacteraceae (7-12%) Planctomycetaceae (8-9%) and Hahellaceae (8.2-15%). It appears that increasing copper concentration caused a decrease in Hahellaceae and Planctomycetaceae OTUs and a subsequent increase in OTUs associated with Flavobacteriaceae and Rhodobacteraceae (Figure 6.7 C).

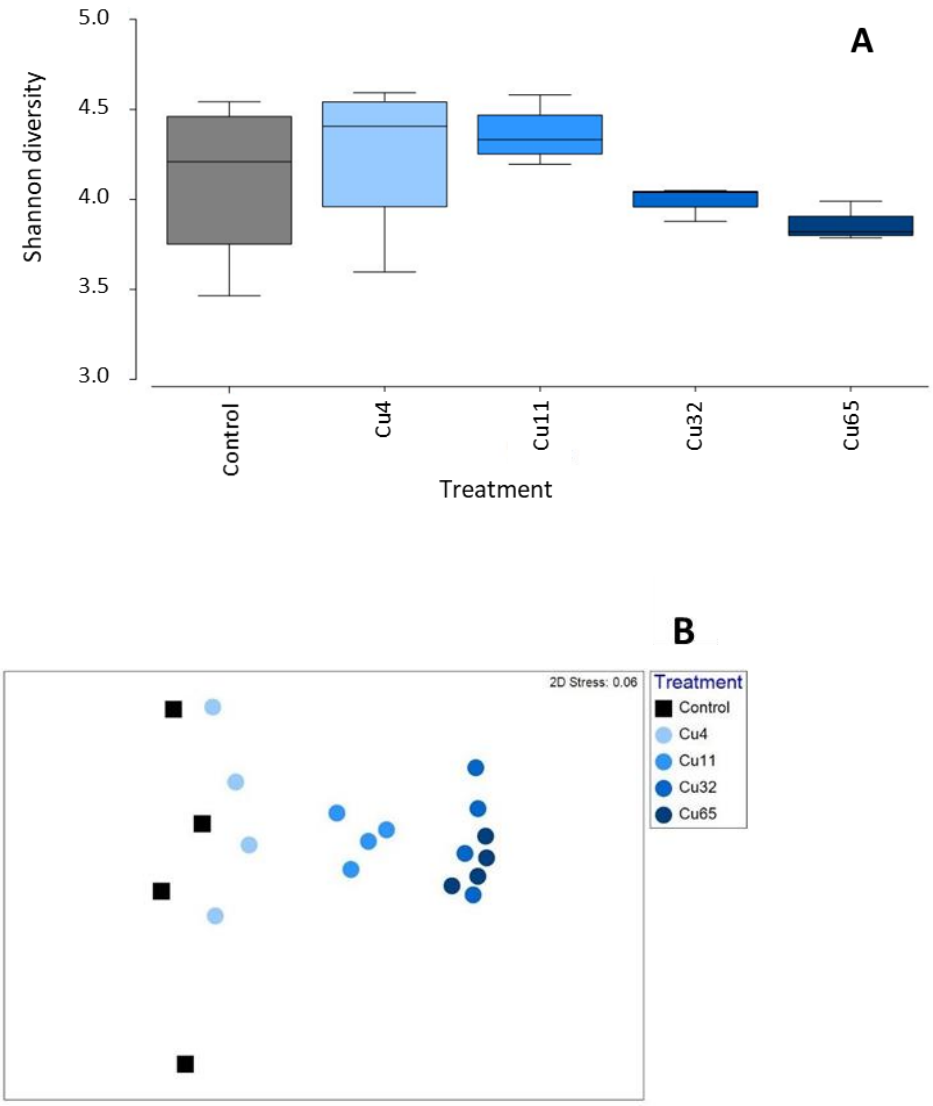



Figure 6.7. The effect of copper on the bacterial community composition of the coral microbiome following 36 to 96-h exposure. A) Boxplots showing the variation in Shannon diversity (mean and variation of 4 replicates) across control and copper treatments. B) Non-metric multidimensional scaling plot showing the relative similarity of the 16S community composition. The (dis)similarity between control and copper treatments was determined by Bray-Curtis similarity. Each point represents one individual replicate from each treatment. C) Shade plot demonstrating the changes in taxa in response to increasing concentrations of nickel, note, for simplicity, only the top 10 taxa are shown. Taxonomic level = Family, OTU = Operational Taxonomic Units, ukF= unknown Family, Family level not identified in classification. Cu4 = 4 µg Cu/L and so on. Metal concentrations are measured dissolved values in µg/L. Data were transformed by square root transformation.

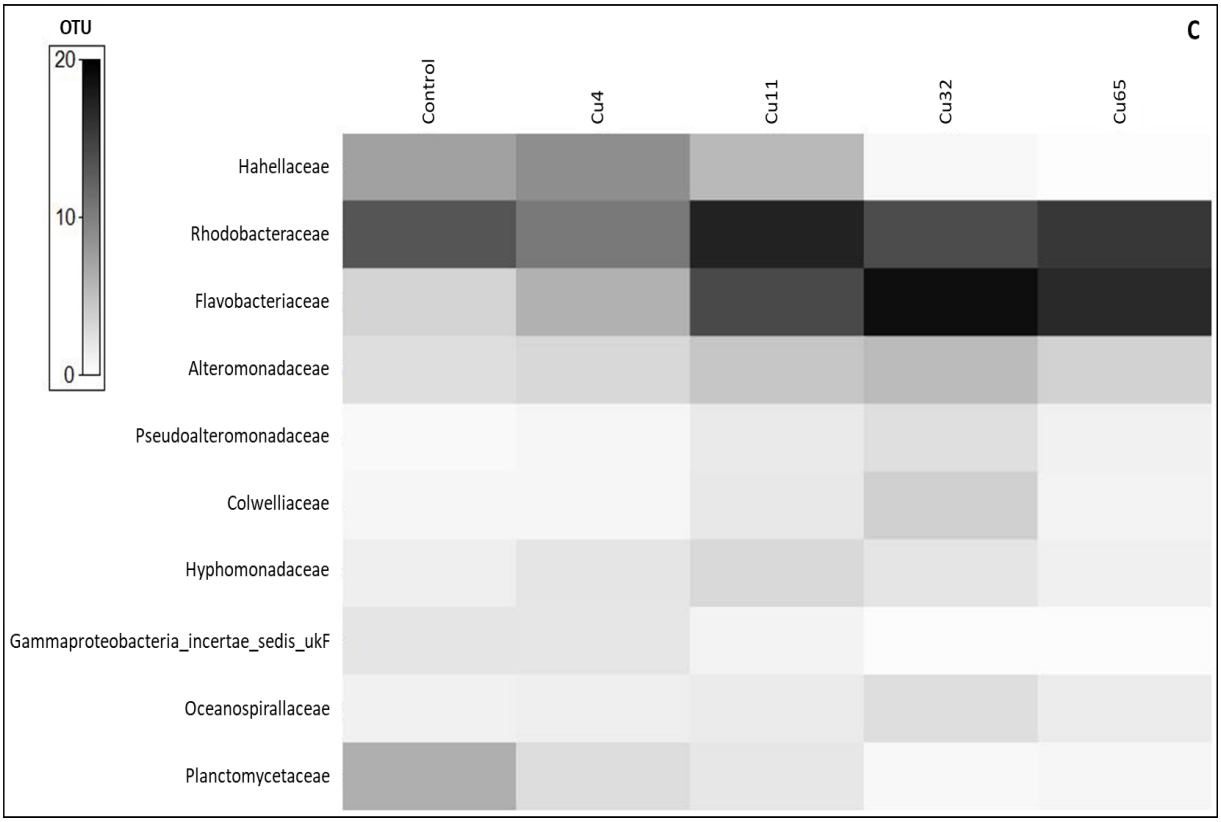



Figure 6.7. Continued.

## 6.4. Discussion

### 6.4.1. Response of corals to nickel and copper exposure

In this study, bleaching was observed at high nickel and copper concentrations (≥11 µg Cu/L and ≥ 470 µg Ni/L). While background concentrations of metals in tropical marine waters are typically <1 µg/L for copper and <5 µg/L for nickel (Apte et al., 2006), in heavily polluted waters, concentrations of dissolved nickel can exceed 1000 µg/L (Pyle and Couture, 2012), and therefore corals may be at risk in some polluted environments. A study around New Caledonia, an area of prominent nickel mining activity measured nickel and copper concentrations ranging from < 0.1 – 11 µg Ni/L and <0.1 – 1.6 µg Cu/L (Moreton et al., 2009).

Although bleaching was observed, coral tissues and polyps remained intact at the lowest copper concentration (4 µg Cu/L). Complete bleaching and polyp retraction was observed at the highest copper concentrations (32-65 µg Cu/L) (data not shown). Effects of copper on *A. muricata* were seen at a lower concentration range compared to previous studies. Past studies have investigated the effects of other metals (copper, cadmium and lead) on another scleractinian coral, *Poccillopora damicornis.* Mitchelmore et al. (2007) found that 50 µg/L of copper and cadmium (exposed individually for 14 days) did not cause bleaching but rather tissue sloughing in *P. damicornis*, where the entire coral tissue and *Symbiodinium* together separated from the skeleton and resulted in coral death (Mitchelmore et al., 2007). Hedouin et al. (2016b) found that survival of adult *P. damicornis* decreased by 50% (LC50) between 175 - 250 µg Cu/L and 477 – 742 µg Pb/L, respectively.

### 6.4.2. Metal uptake and distribution on corals

With increasing concentrations of nickel and copper in seawater, the metal concentrations in coral tissues (algal symbiont and host tissue) also increased. It is possible that the increased metal concentrations measured in the tissues represent the loosely bound metal, or the fraction of metals adsorbed to the coral surface as opposed to absorbed internally,

especially for those samples removed after a 36-h exposure, which were not rinsed with seawater.

Past studies have demonstrated that *Symbiodinium* play an important role in the accumulation and regulation of trace metals in cnidarian hosts (Hardefeldt and Reichelt-Brushett, 2015; Reichelt-Brushett and McOrist, 2003). *Symbiodinium* preferentially accumulated zinc over the host, the anemone *Exaiptasia pallida*, after a 32-d chronic exposure (Hardefeldt and Reichelt-Brushett, 2015). The density of *Symbiodinium* influenced zinc loading in anemones (Hardefeldt and Reichelt-Brushett, 2015). An earlier study by Reichelt-Brushett and McOrist (2003) found that in corals sampled from the Great Barrier Reef (Qld, Australia), most metals (including nickel and copper) accumulated in higher concentrations in *Symbiodinium* than coral tissue. The authors concluded that the loss of *Symbiodinum* (i.e. bleaching) during stress events may play an important role in regulating metal loads in corals (Reichelt-Brushett and McOrist, 2003). Conversely, Mitchelmore et al. (2007) found no significant difference in the partitioning between the algal or animal fractions in the coral *P. damicornis* when exposed to copper or cadmium for 14 days. These studies highlight the importance of understanding the uptake and distribution of metals in host tissues and endosymbionts.

### 6.4.3. Spatial distribution of nickel in coral fragments

Past studies investigating metal accumulation in coral tissues utilised air blasting or water-pik to remove the tissue layer from the skeleton for acid digestion and analysis (Esslemont, 2000; Reichelt-Brushett and McOrist, 2003). Air blasting is an efficient means of tissue collection, however, the tissue associated with the internal section of the polyp may not be collected. Therefore, other techniques are required to measure metal accumulation inside the coral polyps. Analysis of sectioned coral fragments with laser ablation ICP-MS (LA-ICPMS) and µ-PIXE were trialled to determine if nickel could be detected in the polyps of coral fragments. While quantitative data could not be obtained, elevated nickel concentrations were detected around the polyps in all coral fragments exposed to nickel.

Additionally, as nickel exposure concentration increased, the nickel in the coral fragments also increased (Appendix E, Figure E3 and E4). However, there is still a question around whether nickel is surface- adsorbed or absorbed (i.e. bioaccumulated) metal. To improve analysis of metal accumulation with techniques such as LA-ICPMS and µ-PIXE more work is required to investigate rinsing techniques to remove loosely bound metal (e.g. EDTA rinses) and optimal preservation methods (particularly to keep the tissues and skeletons intact). Traditionally, these techniques are used to analyse hard tissues, i.e. skeletons (Runnalls and Coleman, 2003), and further complications arise when attempting to incorporate analysis of both the hard and soft tissue components (Limbeck et al., 2015). Both techniques show promise for future analysis, particularly where there is interest in understanding metal accumulation and distribution in coral polyps.

### 6.4.4. Changes in the coral microbiome

The findings clearly demonstrated that nickel and copper exposure caused significant changes to eukaryote community structure of the microbiome of *A. muricata*. There was a decline in metazoans for both nickel and copper, including Chromadorea, as well as diatoms and brown algae including Bacillariophyaceae (for copper only) and Phaeophyceae. The role of microalgae, other than dinoflagellates in coral microbiomes has yet to be elucidated, however, it is possible that diatoms and brown algae could also influence host-associated microbiomes by releasing carbon-containing photosynthates that fuel microbial metabolism (Bourne et al., 2013). Interestingly, for both copper and nickel, there was an increase in green algae (Chlorophyaceae). This possibly reflects the tolerance of Chlorophyaceae species to metal exposure, which has been demonstrated previously (Levy et al., 2007). Additionally, chlorophytes have been shown to increase in tropical sediments in response to anthropogenic inputs (Graham et al., 2019). For both nickel and copper, there was no significant change in the OTUs associated with Dinophyaceae which includes the algal endosymbiont, *Symbiodinium*. While bleaching was observed, indicating a loss in *Symbiodinium*, it was expected that this would be reflected in the metabarcoding data,

however, the eukaryote dataset was analysed based on presence/absence and not relative abundance. Future work should include *Symbiodinium* density counts, and *Symbiodinium*-specific primers to investigate changes at a finer level of taxonomic resolution and abundance via the use of quantitative PCR.

The majority of coral microbiome studies have focused on changes on the bacterial or prokaryote component and less so on the eukaryotes, unless studying the algal endosymbionts, *Symbiodinium.* In a study on the effects of multiple stressors (tested individually), increased temperature, reduced pH, elevated nutrients and dissolved organic carbon (DOC), Thurber et al. (2009) assessed changes in other eukaryote groups, as well as Alveolata (*Symbiodinium*), including Fungi, Metazoans, Rhodophyta (red algae) and Heterokontophyta (diatoms and brown algae). All stressors caused an increase, compared to control conditions, in Fungi, Metazoans, Rhodophyta and Heterokontophyta. It is not unexpected that primary producers such as Rhodophyta and Heterokontophyta would be stimulated by increased nutrients and carbon source. It is possible that an increase in Fungi was indicative of a disease-like state in the coral microbiome (Thurber et al., 2009).

The relative abundance of bacteria and community composition of the coral microbiome were not affected by increasing nickel concentration. The overall community composition changed significantly with increasing copper and there was a decrease in relative abundance of Planctomytaceae and Hahellaceae with a subsequent increase in Flavobacteriaceae and Rhodobacteriaceae. The increased presence of these bacterial taxa in the *A. muricata* microbiome exposed to copper could indicate that the corals were stressed, potentially unable to regulate their microbiome and therefore became dominated by opportunistic and pathogenic taxa. Research into the bacterial genus *Endozoicomonas,* identified in this study under the family Hahellaceae, showed that they play a key role in coral microbiomes and participate in host-associated protein and carbohydrate transport and cycling (Neave et al., 2016). There was a significant decrease in Hahellaceae with increased copper exposure and this is a stress response consistently observed in other studies

(McDevitt-Irwin et al., 2017). Studies have shown that reduced pH reduces abundance of *Endozoicomonas* in corals (Morrow et al., 2015; Webster et al., 2016). Ziegler et al. (2016) also observed a decrease in *Endozoicomonas* in coral microbiomes in response to sedimentation and sewage disposal in the Red Sea. The decline of *Endozoicomonas* in coral microbiomes in response to stress, as demonstrated here and in past studies, could be problematic for corals given the role this bacteria plays as a beneficial symbiont (McDevitt-Irwin et al., 2017; Neave et al., 2016).

Corals exposed to stressors may have a decreased ability to regulate their microbiomes and so allowing the increased presence of potentially pathogenic and opportunistic microbial taxa (McDevitt-Irwin et al., 2017). In a literature review on the response of coral-associated bacteria to stressors including climate change, water pollution and overfishing, McDevitt et al. (2017) identified several taxa associated with stressed corals. These included *Vibrionales, Flavobacteriales, Rhodobacteriales, Altermonodales, Rhizobiales, Rhodospirillales* and *Desulfovibrionales*. Meron et al. (2011) found that reduced pH increased the presence of *Rhodobacteriales* in the coral microbiome of *Acropora eurystoma*. Gignoux-Wolfsohn and Vollmer (2015) found that corals with white-band disease (caused by bacterial pathogens) had a larger percentage of OTUs belonging to *Flavobacteriales.* In the coral *Pocillopora verrucosa, Flavobacteriales* was an indicator taxa for corals collected adjacent to municipal wastewater outfall areas (Ziegler et al., 2016).

### 6.4.5. Results in the context of overall health and function of corals

This study demonstrated that exposures to nickel and copper cause changes to the structure of the microbiome in the reef building coral *A. muricata*. The resulting imbalance in the microbiome could lead to functional changes and facilitate disease development or alterations in metabolism and immunity that lead to bleaching, necrosis and ultimately coral death (Glasl et al., 2017). While structural changes were observed in the coral microbiome there was a simultaneous increase in the tissue concentrations for both nickel and copper, and bleaching, albeit at the higher test concentrations. At lower concentrations, where

bleaching was minimal or had not occurred, there were slight changes in the microbiome community structure. Increases in the number of treatment replicates could improve our ability to detect changes in the microbiome community structure, however, this is a limitation of microcosm studies where large sample sizes are often not possible (Chariton et al., 2016; Ho et al., 2013). Another limitation to microcosm studies is that the experimental set up is essentially a closed system and the DNA of dead organisms could be attenuating our capacity to detect changes in the community. The use of RNA would reveal who in the community is living and functional (Chariton et al., 2014).

Given that microorganisms respond rapidly to environmental conditions, it is vital to understand how the microbiome of corals responds to a range of natural and anthropogenic stressors. It is possible that the microbial community of corals could be used as an early warning indicator to identify stressed ecosystems prior to more advanced visual cues such as tissue necrosis or bleaching, which are only evident when coral health has already become compromised (Glasl et al., 2017).

## 6.5. Conclusion

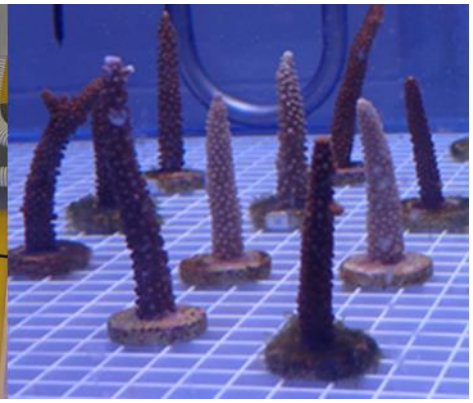
Coral reefs are subjected to numerous global and local stressors including impacts from climate change (increased sea surface temperature, ocean acidification), invasive pest species (e.g. Crown of Thorns Starfish), total suspended solids, oil spills, pesticide run-off as well as metals. In many tropical regions, local stressors are likely to be associated with mining activities. To mitigate this risk requires an understanding of the potential impacts of metals on ecosystems. Multiple lines of evidence were used to investigate the effects of copper and nickel, separately, on the common branching coral *Acropoa mucricata*. Following 36 – 96-h exposure, bleaching was observed at copper concentrations ≥11 µg Cu/L, and nickel concentrations ≥470 µg Ni/L. As metal concentrations in the seawater increased, there was an increase in copper and nickel concentrations in the coral tissues. Significant changes were seen in the microbiome community structure. Nickel concentrations of 470 µg Ni/L altered the eukaryotic communities of the coral microbiome. For copper, significant

differences in both the eukaryotic and bacterial communities were observed at ≥ 11 µg Cu/L. Importantly, there was a loss in Hahellaceae, a known beneficial bacteria in coral microbiomes, when exposed to copper. An increase was seen in Flavobacteraceae and Rhodobacteraceae, two taxa which are believed to be indicative of stressed coral microbiomes. Collectively, the findings show that metal exposure not only has the potential to cause bleaching in corals, but also, the capacity to alter the microbiomes which are inherently linked to coral health via a range of symbiotic processes. Given the pivotal roles corals play in tropical ecosystems, considerably more research is required to determine how metals alter these coral holobionts, and to ensure that WQGs are adequate for their protection. Future work should utilise lower test concentrations, investigate other physiological endpoints, e.g. photosynthetic efficiency and *Symbiodinium* density and include field studies to investigate the effects of metal contaminants in the environment on corals and their microbiomes. In the environment, coral reefs are exposed to multiple stressors, including suspended sediments and metal contaminants. The impact of suspended sediments and nickel-contaminanted sediments on corals and their microbiome are investigated in the following chapter.

# 7. CORAL MICROBIOMES ALTERED BY EXPOSURE TO SEDIMENT AND NICKEL

## Context Statement

The previous chapter presented findings on the effects of dissolved nickel and copper to the adult coral *Acropora muricata* and its microbiome. In the context of mining activities, there is the potential for corals to be exposed to a combination of sediments and metal contaminants. The aim of this chapter was to investigate the effect of nickel, nickel contaminated sediments, and sediments alone, on the coral microbiome. The chapter contributes an understanding of the role of the coral microbiome during exposure to stressors[6].



[6] The research presented in this chapter was designed as part of my thesis and was done in collaboration with Megan Gillmore (PhD student supervised by Dianne Jolley University of Wollongong and Lisa Golding, CSIRO). This was a large and intensive experiment, Megan and I worked together to address our individual research questions. Megan collected, prepared and analysed the sediments. I assisted in sieving and preparing the sediments for the experiment. I organised access to the National Sea Simulator and equipment required for the experimental set-up. Megan and I conducted the experiment together with assistance from Dianne Jolley, Jenny Stauber, Marc Long and Lisa Golding. The analysis presented in my thesis is focussed on the effects of sediment and metal exposures on the coral microbiome. Megan's thesis will present a more in-depth analysis of the sediment properties and the physiological effects on corals.

## 7.1. Introduction

Increased mining activities in the SEAM region predominantly occur on small island nations where large open-cut mines are common (Fernandez et al., 2006). Open-cut mining results in the removal of vegetation causing an increase in soil erosion that transports terrigenous material and associated metals into the marine environment (Fernandez et al., 2006; Heintz et al., 2015). In New Caledonia alone, it has been estimated that 300 million $m^3$ of soil materials have been mobilised and transported into the fringing coral reef (Fernandez et al., 2017). Metals including nickel, cobalt, chromium, iron and manganese are associated with these sediments and can also be transported into coastal waters (Fernandez et al., 2017, 2006).

The impacts of increased sediments are of serious concern, globally, with an estimated 25% of the world's coral reefs under threat from increasing exposure to sediments (Haywood et al., 2016). Suspended sediments can have deleterious impacts on benthic, sessile organisms, such as corals, by reducing light penetration (required for algal endosymbionts), clogging filtering and feeding apparatus, smothering/burying, increasing energy expenditure to remove sediment, and reducing vitality which increases the vulnerability of corals to pathogens (Bessell-Browne et al., 2017; Fraser et al., 2017; Haywood et al., 2016; Pollock et al., 2014). Increased sedimentation and turbidity as a result of dredging increases the prevalence of disease in corals (Pollock et al., 2014), hinders coral fertilisation and settlement (Ricardo et al., 2016, 2017) and causes mortality in adult corals (Bessell-Browne et al., 2017).

The sensitivity of corals to suspended sediment is wide ranging (Erftemeijer et al., 2012). A review by Erftemeijer et al. (2012) collated studies on sediment disturbances on coral reefs and found that coral reef ecosystems can tolerate <10 mg/L total suspended sediment (TSS) concentrations (in pristine offshore environments) to >100 mg TSS/L in nearshore environments. Some coral species can tolerate exposures to high suspended sediments for several weeks, while others die after short-term exposures (days) to concentrations as low

as 30 mg TSS/L (Erftemeijer et al., 2012). In tropical environments, periods of high rainfall may lead to an increase in sediment loads for short periods of time, resulting in pulsed exposure events. Background TSS concentrations are typically <5 mg/L, but adjacent to mining and dredging operations concentrations frequently reach 20 – 30 mg/L, and have been reported as high as 500 – 1000 mg/L for very short periods (Nelson et al., 2016; Reichelt and Jones, 1994; Thomas et al., 2003).

Most studies on sediment and turbidity impacts on corals have focussed on the physical effects on corals as a result of dredging activities. However, an additional impact may occur through the release of metal contaminants from suspended sediments (Fernandez et al., 2017; Haywood et al., 2016; Prouty et al., 2013). Prouty et al. (2013) found elevated metal uptake (of lead, zinc and manganese) in corals adjacent to a marine landfill site in Bermuda and linked this to the mobilisation of metals from resuspension of the surrounding sediments. In New Caledonia, nickel concentrations in suspended sediment in coastal environments can be >7000 mg Ni/kg (Fernandez et al., 2006), and marine organisms including cephalopods, bivalves and ascidians in areas adjacent to nickel mining activities also contained elevated nickel-tissue concentrations (Bustamante et al., 2000; Hedouin et al., 2009; Monniot et al., 1994). Therefore, potential impacts on coral reefs can result from the physical effects of suspended sediments, as well as the contaminants associated with sediments. To date, there has been relatively little research investigating the effects of suspended sediments and associated metal contaminants on adult corals. Furthermore, as discussed in Chapter 6, there is a limited understanding on how these impacts affect adult corals and their associated microbiome.

The aim of this study was to investigate the effect of suspended and nickel-contaminated sediments on the adult coral *Acropora muricata* and its microbiome. Three sediment types were tested: (i) clean sediment (9 mg Ni/kg); (ii) the same clean sediment spiked with nickel under laboratory conditions, referred to as Ni-sediment (6300 mg Ni/kg); and (iii) a sediment collected from the field known to be enriched in nickel, referred to as Field-sediment (240 mg

Ni/kg). The concentration of the Ni-sediment was chosen to reflect environmentally realistic concentrations of nickel found in sediments around mining locations in SEAM (Fernandez et al., 2006). Dissolved nickel treatments were included to enable comparisons with the experiment presented in Chapter 6 and also to provide a reference for the effects of dissolved nickel fraction that may occur following nickel dissolution from the Ni-sediment treatment (280 ± 40 µg Ni/L measured in the tanks). Sediment treatments were tested at two concentrations: 5 mg TSS/L, representative of background TSS concentrations in tropical regions and 30 mg TSS/L representing elevated levels detected around mining and dredging operations (Haywood et al., 2016).

The focus of this chapter was to investigate changes in the microbiome community of corals exposed to sediments and nickel-contaminated sediments. The coral microbiome was described in Chapter 6, Section 6.1. Changes in the eukaryote, prokaryote and *Symbiodinium* communities were investigated to determine if these components of the microbiome were affected by exposure to sediments, and if changes in the community structure could affect the overall health and functioning of adult corals. This study was part of a larger experiment which assessed multiple endpoints including algal endosymbiont (*Symbiodinium*) density, bleaching, and metal accumulation in coral tissues and *Symbiodinium* (Gillmore in prep).

## 7.2. Methods

### 7.2.1. General laboratory techniques and reagents

All glassware and plastic containers used in the tests were prepared as per Chapter 3 section 3.2.1, and soaked in seawater as described in Chapter 5, Section 5.2.1.

A nickel stock solution of 1 g Ni/L was made as per Chapter 3, Section 3.2.1. This stock was diluted in seawater for the dissolved nickel treatments.

Physico-chemical parameters (pH, DO, conductivity, salinity) were measured using a Multi probe (HQ40d Multi-Hach), calibrated following instructions from the manufacturer. Analysis

of total and dissolved metal sub-samples and DOC were analysed as described in Chapter 3, Section 3.2.4.

### 7.2.2. Sediment collection, preparation and analysis

Sediments were collected and spiked as per (Gillmore, in prep). Briefly, the field sediment (enriched in nickel) was collected from Saunders Beach (Yabulu, 19°10'14.5"S 146°37'25.4"E, Qld, Australia) located adjacent to a nickel refinery. Relatively uncontaminated sediment was collected from Hartleys Creek, (Wangetti, 16°39'17.3"S 145°34'04.6"E, Qld, Australia) and was used as the clean control sediment, and a portion of this was spiked with nickel to represent a worst-case scenario, the Ni-sediment. For both sediments, the surface sediment (upper 10 cm), was collected using a clean plastic hand trowel and transported back to the laboratory, sieved to remove particles >180 μm, and stored at 4°C. The high nickel treatment was prepared using a two-part spiking procedure. Nickel chloride ($NiCl_2.6H_2O$) dissolved in filtered seawater (Chapter 3, Section 3.2.1), was thoroughly mixed into the sediment by hand and then allowed to equilibrate at room temperature for 10 weeks, with twice weekly mixing by hand (Gillmore in prep., Brumbaugh et al., 2013; Simpson et al., 2013). The spiked sediment was then diluted 6:1 with clean control sediment and allowed to equilibrate for a further 2 weeks at 4°C (Gillmore in prep).

Total recoverable metals in the sediments were determined after aqua-regia digestion (3:1 ratio of concentrated $HNO_3$ and HCl, respectively) (Tracepur, Merck) in 50 mL Greiner tubes containing 0.35 ± 0.05 g of oven dried (110°C for >24 h) sediment. Digestion was achieved after microwave heating (CEM MARS) at 80°C for 1.5 h. Dilute acid-extractable metals were determined by reacting cold 1 M HCl with 0.5 ± 0.05 g wet sediment in a sediment:acid ratio of 1:50 for 1 h. For both analyses, all samples were measured in duplicate. The analysis of method blanks and certified reference materials AGAL-10 (National Measurement Institute, Australian Government) were also processed as part of routine quality control procedures. The metal concentrations of the digests were measured using ICP-AES (Chapter 3, Section 3.2.4). Sediment grain size was determined using laser diffraction techniques (Mastersizer

2000, Malvern Instruments Ltd, UK). Total carbon was determined on dried (60°C for ≥ 24 h) and finely ground samples by high-temperature combustion in an atmosphere of oxygen using a Leco TruMAC instrument (LECO Corporation, USA). Carbon was converted to $CO_2$ and determined by infrared detection. Total inorganic carbon was determined by reacting the sample with acid in a sealed container and measuring the pressure increase. The sample, containing no more than 0.8 g $CaCO_3$ equivalent, was weighed into a 250 mL glass bottle, combined with 8 mL 3 M HCl and 3% $FeCl_2$ added and the bottle sealed. The contents were mixed intermittently over 1 hour and the pressure in the bottle measured by piercing the septum with a needle attached to a pressure transducer. Total organic carbon (TOC) was determined by subtracting the total inorganic carbon from the total carbon (Gillmore in prep). Sediment properties are provided in Table 7.1.

### 7.2.3. Species collection and maintenance

Coral experiments carried out at the National Sea Simulator (SeaSim), Australian Institute of Marine Science (AIMS), Townsville, Australia. Coral collection and maintenance is described in Chapter 6, Section 6.2.2. The scleractinian branching coral, *A. muricata*, was collected by SeaSim staff on 16$^{th}$ June 2017 from Trunk Reef (18° 18.173'S, 146° 52.153'E), at 3 – 5 m depth, Great Barrier Reef, Queensland, Australia (GBRMPA Permit number G12/35236.1). Six colonies of *A. muricata* were fragmented (into 5-8 cm fragments) on board the boat and mounted onto aragonite plugs, using super glue (XTRA Loctite super glue, Loctite Australia Pty Ltd), and maintained in 60-L aquaria with flowing seawater (5 L/min) from the collection point until returned to the SeaSim aquaria on the 19$^{th}$ June 2017.

Table 7.1. Characteristics of sediments following preparation for experimental treatments, all sediments were sieved to < 180 µm. Values are rounded up to two significant figures.

| Sediment | Particle size, (%) | | | Total organic carbon (%) | Dilute-acid[a] extractable metals (mg/kg) | | | Total recoverable metals (mg/kg) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | <4 µm | 4-63 µm | >63 µm | | Ni | Fe | Mn | Ni | Fe | Mn |
| Clean-sediment | 6 | 82 | 12 | 3.5 | 1 | 650 | 7 | 9 | 14000 | 120 |
| Ni-sediment | 6 | 82 | 12 | 3.5 | 6100 | 540 | 6 | 6300 | 15000 | 120 |
| Field-sediment | 7 | 82 | 11 | 7.7 | 60 | 11000 | 33 | 240 | 23000 | 100 |

[a] 1M HCl, 60 min, room temperature

### 7.2.4. Toxicity testing with adult corals

The toxicity tests commenced on the 4th August 2017, approximately 6 weeks after coral collection and acclimation to aquaria conditions. There is no established protocol for acclimating adult corals prior to testing in aquaria conditions, however, the health of the corals was noted by observing the colour of the fragments and the presence of skeletal growth around the base of the fragments on the aragonite plugs.

The experimental set-up followed that described in Bessell-Browne et al. (2017). Experimental chambers were 115 L clear PVC tanks each with an inverted triangular base to reduce sediment deposition on horizontal surfaces. Tanks were supplied with filtered (0.04 µm) seawater at a rate of 400 mL/min (per tank) to ensure six complete turnovers per tank/day. Water circulation in the tanks was maintained by a magnetically driven centrifugal pump that forced water flow up from the central base of the tank, also reducing sediment deposition (Figure 7.1). Sediment treatment tanks were periodically dosed from a concentrated header tank of the stock sediment slurry. Dosing was controlled by a programmable logic controller (custom control logic Siemens S7-1500 PLC) that controlled solenoid valves connected to the stock sediment tanks via a high velocity loop powered by an air diaphragm pump (Bessell-Browne et al., 2017). Turbidity in sediment tanks was measured by turbidity meters connected to the tanks (Appendix F, Figure F1). Light was provided above each tank using LED lights (Al Hydra FiftyTwo™ HD, Aquaria Illumination, IA, US). A light meter (spherical quantum sensor, Li-COR LI-193) was attached underwater at the same level of the coral fragments, in one replicate tank per sediment treatment (Appendix F, Figure F2). Sediment treatments consisted of a control sediment which is here on referred to as clean-sediment (9 mg Ni/kg), Ni-sediment (6100 mg Ni/kg) and field-sediment (240 mg Ni/kg). All three sediments were tested at ~5 and ~30 mg TSS/L, with three replicate tanks per treatment. In addition, there were seawater-only treatments (no sediment dosing) which consisted of a control (no nickel) and 200 µg Ni/L and 400 µg Ni/L (nominal) treatments; each in duplicate. Two header tanks contained concentrated nickel

solutions (500 and 1000 mg Ni/L in filtered seawater) that were supplied to the treatment tanks at a rate of 0.3 mL/min to achieve the desired nominal concentrations of 200 µg Ni/L and 400 µg Ni/L. Twenty coral fragments were placed in each tank 24 h prior to starting the exposure to allow the corals to acclimate to the test conditions. The exposure ran for 7 days, after which dosing of all treatments ceased and the tanks were circulated with clean filtered seawater. Corals were maintained in tanks for a recovery period for a further 7 days. Samples were collected on Day 0 (baseline, six representative coral fragments sampled), Day 4, Day 7 (during exposure) and Day 14 (recovery). Two coral fragments were sampled from each replicate tank at each time point, Day 7, 7 and 14 (Figure 7.2). No food was provided during the entire experimental period. Physico-chemical parameters (temperature, pH, dissolved oxygen, conductivity, salinity) were measured in all tanks on Days 0, 2, 4, 6, 8, 10, 12 and 14. Sub-samples for total and dissolved metals were collected daily from each tank during the exposure period, and every second day during the recovery period. Details on the experimental set-up and conditions are provided in Table 7.2.

To verify the automated sediment dosing system, TSS concentrations in each tank (of sediment treatments) were determined (Appendix F, Table F1). Water samples (500 mL) were taken from the tanks (duplicate samples per tank) and filtered through a pre-weighed 47 mm diameter polycarbonate filter paper (0.4 µm). Filter papers were dried at 60°C for at least 24 h and weighed to 0.0001g. This was done on Days 0, 1, 2, 3, 5 and 6.

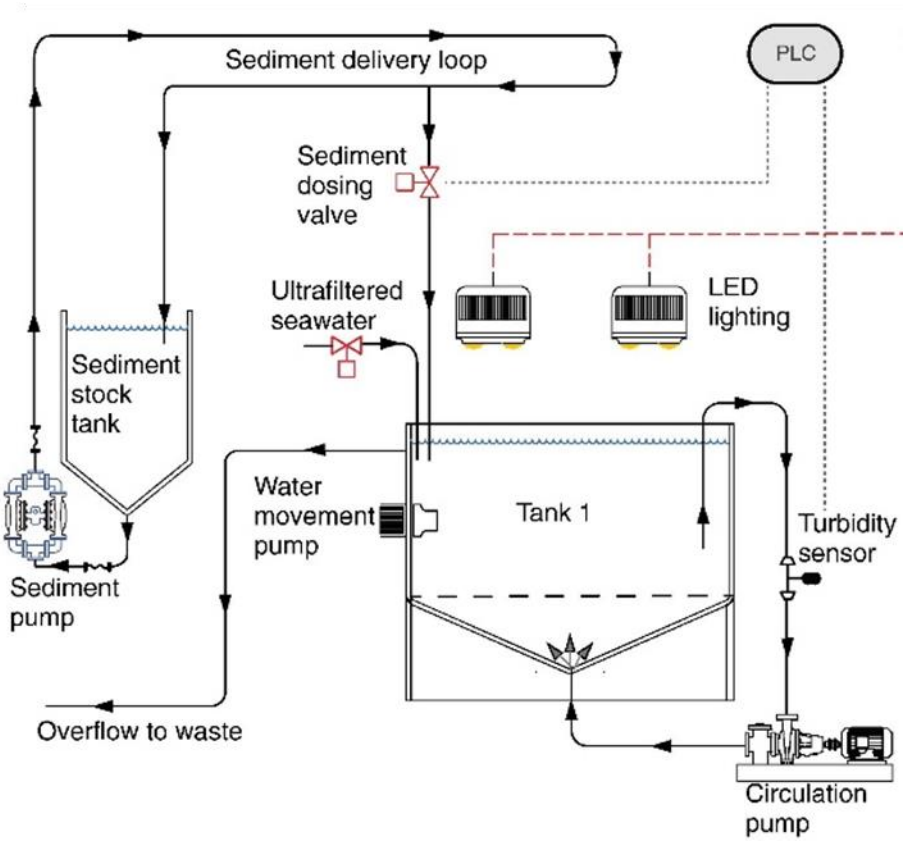
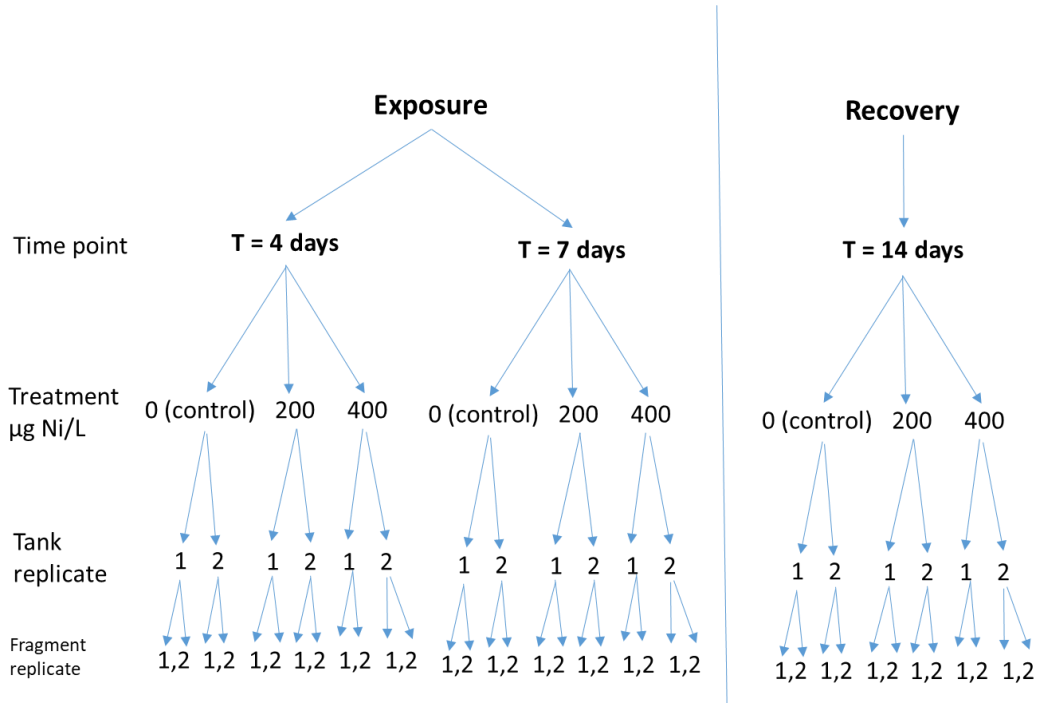



Figure 7.1. The experimental set-up for the exposure of the adult coral *Acorpora mucircata* to dissolved nickel and sediments (from Bessell-Browne et al., 2017). PLC = programmable logic controller.

**A) Dissolved nickel**




**B) Clean and Ni-sediment**

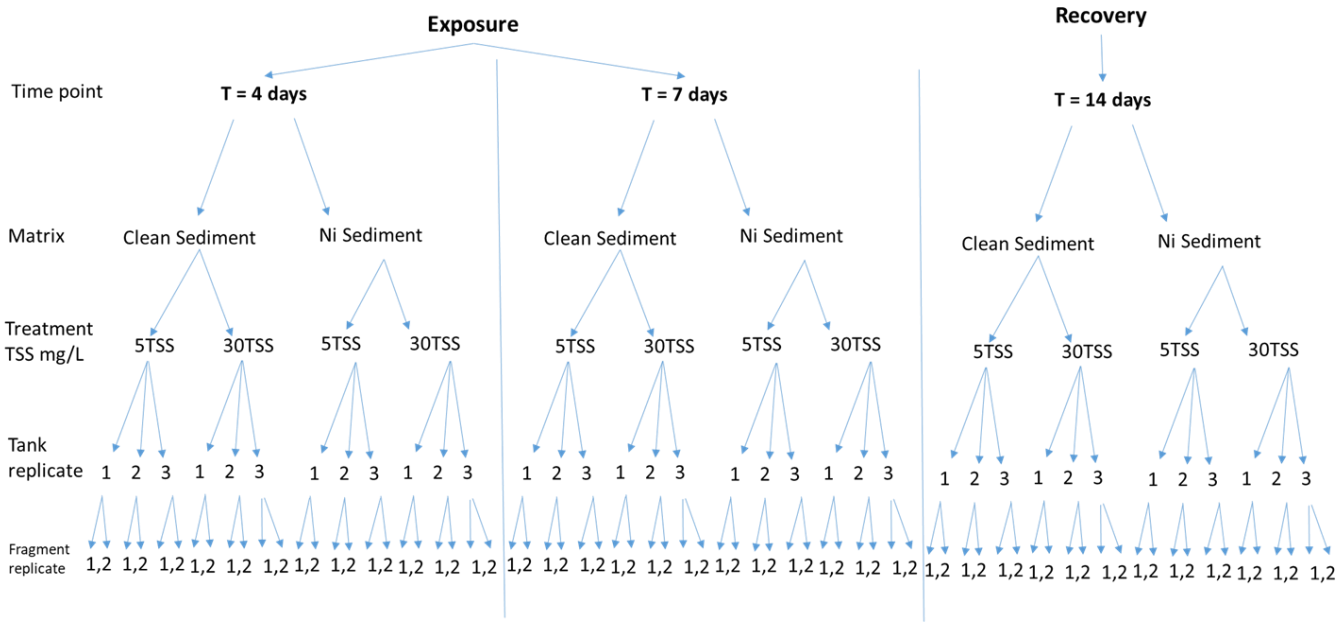



Figure 7.2. Experimental design for each treatment. A) Dissolved nickel, B) clean and Ni-sediment and C) Field sediment

### C) Field-sediment

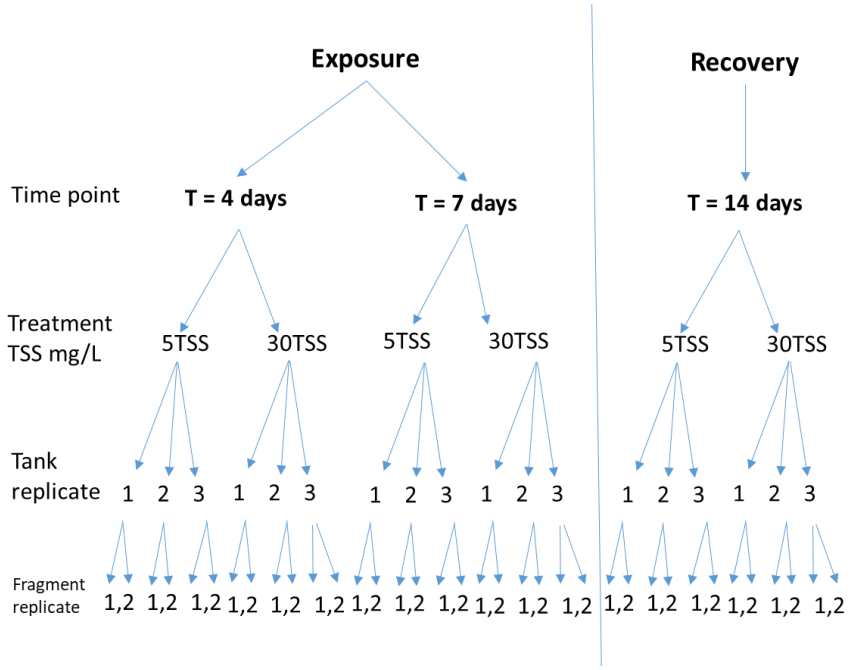



Figure 7.2. Continued.

Table 7.2. Toxicity test conditions and parameters for 96-h exposure with *Acropora muricata*. Mean values ± standard deviation

| **Test conditions/parameters** | |
|---|---|
| Temperature (°C) | 27 ± 0.1 |
| Salinity (‰) | 36 ± 0.3 |
| Conductivity (mS/cm) | 54 ± 0.5 |
| DO (%) | 97 ± 6 |
| pH | 8.1 ± 0.1 |
| Dissolved treatments | 0, 200, 400 µg Ni/L, two replicate tanks per treatment |
| Sediment treatments | 5 and 30 mg TSS/L for each sediment type, three replicate tanks per treatment |
| Light parameters | Low light 6:30 (dawn)<br>Full light 8:00 (max intensity, 250 µmol/m$^2$/s). Irradiance is similar to the expected mean photosynthetically active radiation at the collection site.<br>Low light 16:00 (dusk)<br>Lights off 18:00 |
| Test type | Flow through 400 mL/min, 6 turnovers in each tank per day |
| Test chamber | 115 L PVC tank and lid |
| Test volume | 100 L |
| Test duration | 7-days exposure, 7-days recovery |
| Control/diluent water | 0.04 µm natural filtered seawater |
| Life stage of test organism | Adult, 5-8 cm fragments |

### 7.2.5. DNA extraction amplification and sequencing

Methods followed those provided in Chapter 6, Section 6.2.6. However, at the time of sampling, coral tissues were preserved in RNALater® (Sigma-Aldrich) prior to flash freezing in liquid nitrogen. To remove the RNALater® prior to extraction, an ethanol wash step was required. Samples were gently thawed on ice, centrifuged (max speed for 5 mins at 4°C), the supernatant (RNALater®) was removed using care not to disturb the pelleted sample (i.e. coral tissues); and the pellet was resuspended in ethanol (500 µL, 70%, at -20°C). Samples were centrifuged again, supernatant removed, and samples left to air dry for 2 min to ensure the remaining ethanol evaporated. Then DNA was immediately extracted using the QIAGEN® DNeasy Power Biofilm kit (QIAGEN®, Germany), following instructions from the manufacturer with some modifications (Chapter 6, Section 6.2.6). For the eukaryote community, DNA was amplified using the All18SF (5'-3': TGGTGCATGGCCGTTCTTAGT) and All18SR (5'-3': CATCTAAGGGCATCACAGACC) primers for the V7 region of the 18S rRNA gene (Hardy et al., 2010). For the prokaryote community DNA was amplified using the 515f (5'-3': GTGYCAGCMGCCGCGGTAA) (Baker et al., 2003; Quince et al., 2011), 806r (5'-3': GGACTACNVGGGTWTCTAAT) (Apprill et al., 2015) for the V4 region of the 16S rRNA gene (recommended by Earth Microbiome Project, EMP). For the *Symbiodinium* community DNA was amplified using the internal transcribed spacer 2 (ITS2) rDNA (forward 5′-GTGAATTGC AGAACTCCGTG-3′ and reverse 5′-CCTCCGCTTAC TTATATGCTT-3′) primers (Boulotte et al., 2016). Details on the 18S rDNA and 16S rDNA amplifications (Polymerase Chain Reaction, PCR) of samples are provided in Chapter 6, Section 6.2.6. Amplification of samples using the ITS2 primers also used the Amplitaq Gold 360 Master Mix (Applied Biosystems), and DNA-free water (Millipore®). The PCR for ITS2 primers consisted of 21 µL of water, 25 µL of Master Mix, 1 µL of each of the forward and reverse primers (10 µM) and 3 µL of template DNA. The PCR conditions included denaturing at 95°C for 5 min, annealing (30 cycles) at 95°C for 30 s, 52°C for 40 s, 72°C for 30 s, and extension at 72°C for 3 min.

Following amplification all samples were pooled, each amplicon library (18S rDNA, 16S rDNA, ITS2 rDNA) kept separate, purified and checked using a MultiNA gel as described in Chapter 6, Section 6.2.6. The final pooled amplicon library concentrations were measured on the Nanodrop and sent for sequencing to the Ramaciotti Centre for Genomics (University of New South Wales, Australia). Amplicon libraries were prepared for sequencing using TruSeq PCR-free kit. As the base pair (bp) sizes of the amplicons were different for 18SrDNA (180bp), 16SrDNA (350bp) and ITS2 rDNA (300bp) the libraries were run as three separate Illumina® Miseq sequencing runs, 2x 150bp for 18S, 2x 250bp for 16S and 2x 300bp for ITS2. Raw sequences are available at https://data.csiro.au/dap/landingpage?pid=csiro:35242.

### 7.2.6. Bioinformatics

Sequenced data were processed using a custom pipeline Greenfield Hybrid Amplicon Pipeline (GHAP) which is based around USEARCH tools as discussed in Chapter 6, Section 6.2.7. At the time of processing samples for this study the reference dataset for the ITS2 primers, cited by Boulotte et al. (2016), was no longer available, so a reference set was generated using usearch_global and formatted as required for GHAP (Edgar, 2013). The pipeline is available at https://doi.org/10.4225/08/59f98560eba25 .

### 7.2.7. Statistical analysis

After processing through the bioinformatics pipeline and prior to statistical analyses, data were processed through a final filtering step. For the 18S and 16S data, the highest reads for the positive control operational taxonomic unit (OTU) in samples were 148 and 38, respectively. These two values were used as the cut-off points for filtering the dataset, with 18S rDNA and 16S rDNA OTUs with maximum detections of 148 or 38 reads, respectively, removed, thereby eliminating potential tag-jumped sequences and low quality reads. OTUs which had a match percent of <80% were also removed. The positive controls amplified in the PCR were also used as a check for successful amplification and sequencing and to check for cross contamination in the 18S, 16S and ITS2 libraries. The positive controls and

OTUs were removed from the dataset and this final dataset was used in the statistical analysis. For the 18S rDNA data, all coral OTUs were also removed to focus on the microbiota only. The number of reads and OTUs before and after filtering are provided in Appendix F, Table F2.

For each of the datasets, the correlation between the number of OTUs (i.e. richness) and read count in each sample was determined to identify whether or not rarefying the data was necessary (Egge et al., 2013). For the 18S, 16S and ITS2 datasets, the correlation between richness and counts were 0.06, 0.4, and 0.6, respectively. Therefore, the 18S dataset was not rarefied, while the 16S and ITS2 datasets were rarefied to minimum read counts of 1825 and 105, respectively. Statistical analyses were completed on rarefied and non-rarefied 16S and ITS2 datasets. As there was no difference in the overarching results, the non-rarefied datasets were used for consistency with the 18S dataset. It can also be argued that rarefying data is inappropriate for the detection of differentially abundant species (McMurdie and Holmes, 2014).

The 18S rDNA dataset was transformed to presence/absence prior to computation (Chariton et al., 2015). The 16S rDNA and ITS2 rDNA datasets were initially standardised by the total abundance and then square-root or fourth-root transformed, respectively, to calculate the relative abundances of each OTU across samples. The 18S rDNA dataset was aggregated down to the taxonomic level of class, while the ITS2 dataset was aggregated down to species level and the 16S rDNA dataset was aggregated down to family. All univariate and multivariate statistical analyses were as described in Chapter 6, Section 6.2.8. Non-metric dimensional scaling plots (nMDS) were generated using the bootstrap method to depict relative similarities in community composition between different treatments. Statistical differences between treatments were tested by PERMANOVA ($P \leq 0.05$). Initially, a main test was run with unrestricted permutation of raw data, with 9999 permutations, to identify significant differences and interaction between factors. For the clean and nickel-spiked sediments, there were four factors: matrix (sediment type, 2 levels, fixed), treatment (TSS

concentration, 2 levels, fixed), time point (3 levels, fixed) and tank replicate (nested in matrix and treatment, 3 levels, random). For the dissolved and field sediment treatments, there were three factors: treatment (nickel concentration or TSS concentration, 3 and 2 levels, respectively, fixed), time point (3 levels, fixed) and tank replicate (nested in treatment, 2 levels for dissolved and 3 levels for field sediment, random) (Figure 7.2). After testing for interactions, differences between matrices, treatments and time points were identified by pairwise *a posteriori* tests based on 9999 unrestricted permutations of raw data. Univariate attributes in the prokaryote and *Symbiodinium* microbiome communities, including richness (Margalef, d), evenness (Pielou's, J') and diversity (Shannon, H') were obtained using the DIVERSE function in Primer 7+ statistical package (Plymouth Marine Laboratory, UK). Differences in univariate attributes ($p \leq 0.05$) were determined by one-way ANOVA in NCSS (Chapter 6, Section 6.2.8).

## 7.3. Results

### 7.3.1. Quality control

Physico-chemical conditions were tightly controlled in all tanks throughout the 14-d experimental period (7-d exposure to treatments + 7-d recovery period in seawater only, Table 7.3). Light intensity measurements, detailing diurnal patterns, are provided in Appendix F, Figure F2.

Throughout the experimental period, background concentrations of metals in all tanks remained low (µg/L: Al 4.4, As <0.76, Cd <0.56, Co <0.24, Cr <1.1, Cu 1.1, Fe 0.6, Mn <0.21, Zn 1.0). During the exposure period, the concentrations of dissolved nickel in the seawater-only treatments were close to nominal values, 200 ± 20 µg Ni/L and 420 ± 50 µg Ni/L, respectively, and in the control tanks remained below the LOD (1 µg Ni/L, Table 7.4). The concentrations of dissolved nickel in the clean-sediment treatment tanks and in the field-sediment 5 mg TSS/L treatment tanks were also below the LOD. In the Ni-sediment treatment tanks the dissolved nickel concentrations at 5 mg/L and 30 mg/L TSS were 70 ± 7.4 and 280 ± 40 µg Ni/L, respectively. During the recovery period, this decreased to 2.6 ±

1.0 and 10 ± 8.4 µg Ni/L in the 5 and 30 mg TSS/L Ni-sediment tanks, respectively. Concentrations of dissolved nickel in the field-sediment 30 mg TSS/L tanks were 1.4 ± 0.39 and 0.74 ± 0.28 µg Ni/L, respectively during the exposure and recovery periods (Table 7.4). Dissolved organic carbon concentrations during the exposure period (t=0-7 d) were relatively stable in seawater-only treatments and ranged from 1.1-1.3 mg/L, and in sediment treatments ranged from 1.1-1.5 mg/L (Appendix F, Table F3).

Turbidity remained relatively constant over the exposure period (Appendix F, Figure F1). Total suspended sediment concentrations measured by gravimetric analysis, were also constant and in good agreement with turbidity measurements. Across all measurements the mean (± SD) of the ratio of total suspended sediment concentrations (TSS, mg/L) to turbidity (FNU) was 1.1 ± 0.12 (Appendix F, Table F1). During the exposure period, TSS concentrations were close to the target concentrations, with measured concentrations of 4.4 ± 0.64 mg TSS/L and 29 ± 1.4 mg TSS/L for the 5 and 30 mg TSS/L treatments, respectively.

Table 7.3. Physico-chemical parameters in each tank over the 14-d experimental period (n=192)

| Parameter | Range measured over 14-day period |
|---|---|
| Temperature (°C) | 26.6 - 27.1 |
| Dissolved oxygen (%) | 84 - 118 |
| Dissolved oxygen (mg/L) | 5.6 - 7.5 |
| Conductivity (mS/cm) | 53 - 58.6 |
| Salinity (‰) | 35.3 - 36.6 |
| pH | 7.92 - 8.19 |

Table 7.4. Concentrations of nickel in solution in all treatment tanks during the exposure (t=0 - 7 d) and recovery (t=8 – 14 d). The mean was taken for the replicate tanks for each treatment[a]

| Treatment | Concentration of nickel (µg/L) (mean ± SD) | | No. of replicate tanks |
|---|---|---|---|
| | Day 0 - 7 | Day 8 - 14 | |
| Dissolved (seawater) | | | |
| Control[b] | 0.56 ± 0.06 | 0.56 ± 0.06 | 2 |
| Ni 200 µg/L | 200 ± 19 | 1.7 ± 0.43 | 2 |
| Ni 400 µg/L | 415 ± 52 | 2.8 ± 0.94 | 2 |
| Sediment | | | |
| Clean-sediment, 5 mg TSS/L[b] | 0.56 ± 0.06 | 0.56 ± 0.06 | 3 |
| Clean-sediment, 30 mg TSS/L[b] | 0.56 ± 0.06 | 0.56 ± 0.06 | 3 |
| Ni-sediment, 5 mg TSS/L | 70 ± 7.4 | 2.6 ± 1.8 | 3 |
| Ni-sediment, 30 mg TSS/L | 280 ± 40 | 10 ± 8.4 | 3 |
| Field-sediment, 5 mg TSS/L[b] | 0.56 ± 0.06 | 0.56 ± 0.06 | 3 |
| Field-sediment, 30 mg TSS/L | 1.4 ± 0.39 | 0.56 ± 0.06[b] | 3 |

[a] Samples were taken from each tank every day during exposure (t=0 – 7 d) and every second day (t=8-14 d) during the recovery period, the mean and standard deviation of these measurements is shown

[b] Where values were below the LOD half the LOD is given

### 7.3.2. Effects on the coral microbiome

#### *Changes in the coral microbiome community structure in response to aquaria conditions*

To investigate the effects of aquaria conditions on the coral microbiome, samples were taken at the time of coral collection (i.e. from the field), after 6 weeks of acclimation in the aquaria, and following 24 h in the experimental tanks. There was a significant difference in the prokaryote and eukaryote community structures of coral microbiomes sampled from the field compared to the aquaria and laboratory conditions ($P<0.05$, Appendix F, Table F4, Figure F3), however, the *Symbiodinium* community structure remained similar ($P=0.63$). With respect to the bacterial community, the microbiome of corals sampled from the field had a higher relative abundance of Hahellaceae, and corals sampled in the aquaria and under laboratory conditions had a higher relative abundance of Rhodobacteraceae, Vibrionaceae, Flavobacteriaceae and Flammeovirgaceae (Appendix F, Figure F4). For the eukaryote community of the coral microbiome, aquaria and laboratory conditions resulted in a decrease in the presence of OTUs associated with Ostracoda (seed shrimps), Gregarinasina (intestinal parasites) and Ulvophyceae (green algae), compared to corals sampled in the field. In corals sampled from the aquaria and laboratory, there was an increase in the presence of OTUs associated with diatoms (Mediophyceae and Fragilariophyceae, Appendix F, Figure F4).

#### *Dissolved nickel treatments*

There was no change in richness, evenness or diversity in the *Symbiodinium* (ITS2 rDNA) or prokaryote (16S rDNA) communities of the coral microbiome following exposure to dissolved nickel treatments and seawater (control) over the experimental period (7-d exposure, 7-d recovery, $p > 0.05$).

Irrespective of dissolved nickel concentrations, time (days), or tank (location in laboratory), there was no change in the composition of eukaryotes (18S rDNA) ($P>0.05$, Appendix F, Table F5). *Symbiodinium* (ITS2) communities changed in response to time ($P = 0.0457$,

Appendix F, Table F5). The concentration of dissolved nickel caused no significant changes in the prokaryote (16S rDNA) community composition, however, there was an effect of time and all corals sampled at 14 d had significantly different prokaryote community compositions compared to corals sampled at 4 and 7 d ($P<0.05$, Appendix F Table F6).

### *Field sediment treatments*

There was no change in richness, evenness or diversity of the *Symbiodinium* (ITS2 rDNA) or prokaryote (16S rDNA) communities of the coral microbiome following exposure to field sediments for 7 d and during the recovery period ($p > 0.05$).

Irrespective of concentration of TSS, time (days), or tank (location in laboratory), there was no change in the *Symbiodinium* community structure following exposure to the field-sediment at 5 and 30 mg TSS/L ($P>0.05$, Appendix F, Table F7). The structure of the eukaryote and prokaryote communities of the coral microbiome changed over time and were significantly different at each of the time points (T= 4-, 7- and 14- d) ($P < 0.05$, Appendix F, Tables F7 and F8).

### *Clean and nickel-spiked sediment treatments*

There was no change in richness, evenness or diversity in the *Symbiodinium* (ITS2 rDNA) or prokaryote (16S rDNA) communities of the coral microbiome following exposure to clean- and Ni-sediment at 5 or 30 mg TSS/L over the experimental period (7-d exposure, 7-d recovery, $p > 0.05$). Irrespective of sediment type (clean- vs Ni-sediment), TSS concentration, time (days), or tank (location in laboratory), there was no change in the *Symbiodinium* community structure following exposure to clean- and Ni-sediment at 5 or 30 mg TSS/L ($P > 0.05$, Appendix F, Table F9).

The eukaryote community structure of the coral microbiome changed in response to different sediment types (clean- vs Ni-sediment) ($P = 0.0014$, Table 7.5, Figure 7.3). However, the level of TSS had no significant effect on community composition ($P = 0.0512$, Table 7.5, Figure 7.3). At 14 days (after the 7-d recovery period) there was a significant difference in

the eukaryote community composition in the microbiome of corals exposed to clean- and Ni-sediments (P=0.0024, Table 7.5, Figure 7.4). For the corals exposed to clean sediments, there was a change in the eukaryote community composition of the microbiomes at each of the sampled time points (P<0.05, Table 7.6); however, there was no significant difference in the eukaryote communities of coral exposed to Ni-sediment over the experimental period (P>0.05, Table 7.6). Differences in the clean- and Ni- sediment community compositions were driven by Maxillopoda (19% contribution), Bacillariophyaceae (15% contribution) and Dinophyceae (14% contribution); the richness of all three taxa decreased slightly in the Ni-sediment treatments (Figure 7.4).

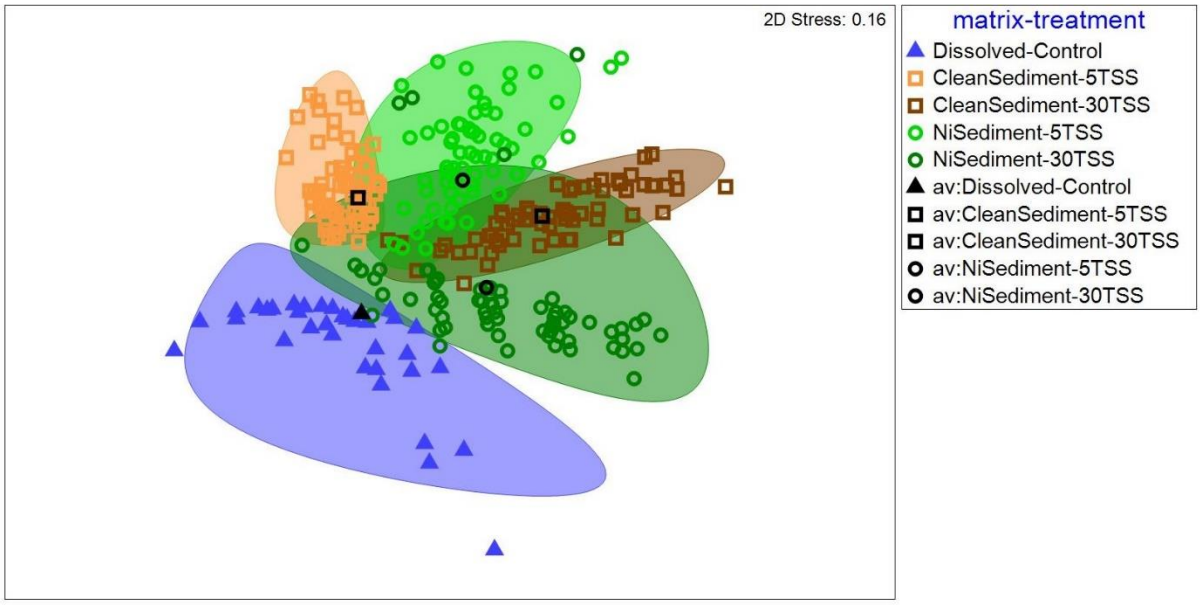



Figure 7.3. Non-metric dimensional scaling plot showing the relative similarity of the eukaryote (18S) community composition of coral microbiomes exposed to clean- and Ni-sediment at 5 and 30 mg/L TSS, relative to the control, corals exposed to natural filtered seawater only. The plot displays the average for each treatment (black shape) and bootstrap averages for each treatment (coloured symbols) and the 95% confidence around each grouping (coloured, shaded region around the mean).

Table 7.5. The effect of clean- and Ni-sediment (matrix) at 5 and 30 mg TSS/L (treatment) on the eukaryote (18S rDNA) community of the coral microbiome. Samples were taken on Day 4 and 7, during exposure and on Day 14, following a 7-d recovery period. Main test (PERMANOVA P = 0.05) investigating the effect and interaction of each of the experimental factors [a]

| Source | df | SS | MS | Pseudo-F | P(perm) | Unique perms |
|---|---|---|---|---|---|---|
| Matrix (Sediment type) | 1 | 1013.8 | 1013.8 | 4.0026 | **0.0155** | 8956 |
| Treatment (mg TSS/L) | 1 | 739.85 | 739.85 | 2.9209 | 0.0512 | 8919 |
| Time (d) | 2 | 2826.3 | 1413.1 | 8.4456 | **0.0001** | 9952 |
| matrix x treatment | 1 | 121.58 | 121.58 | 0.47999 | 0.7362 | 8930 |
| matrix x time | 2 | 1499.3 | 749.65 | 4.4802 | **0.0014** | 9967 |
| treatment x time | 2 | 550.38 | 275.19 | 1.6447 | 0.1533 | 9939 |
| tank (matrix x treatment) | 8 | 2026.3 | 253.29 | 0.47601 | 0.9737 | 9899 |
| matrix x treatment x time | 2 | 750.76 | 375.38 | 2.2434 | 0.0557 | 9957 |
| time x tank (matrix x treatment) | 16 | 2677.2 | 167.32 | 0.31445 | 1 | 9879 |
| Residual | 36 | 19156 | 532.11 | | | |
| Total | 71 | 31362 | | | | |

[a] df = degrees of freedom, SS = sum of squares, MS = mean squares

Table 7.6. The effect of clean- and Ni-sediment (matrix) on the eukaryote (18S rDNA) community of the coral microbiome. Samples were taken on Day 4 and 7, during exposure and on Day 14, following a 7-d recovery period. Pair-wise tests (PERMANOVA P = 0.05) comparing the effect of sediment type and time on the community composition of the coral microbiome.

| Within level of time | t | P(perm) | Unique perms |
|---|---|---|---|
| t=4 d | | | |
| Clean sediment, Ni sediment | 1.58 | 0.0893 | 8963 |
| t=7 d | | | |
| Clean sediment, Ni-sediment | 1.32 | 0.1905 | 8991 |
| t=14 d | | | |
| Clean sediment, Ni-sediment | 3.06 | **0.0024** | 8954 |
| Within level of matrix | | | |
| Clean sediment | | | |
| t=4, t=7 | 2.01 | **0.0291** | 9948 |
| t=4, t=14 | 4.35 | **0.0037** | 9948 |
| t=7, t=14 | 3.41 | **0.0008** | 9938 |
| Ni-sediment | | | |
| t=4, t=7 | 1.33 | 0.2228 | 9965 |
| t=4, t=14 | 1.59 | 0.1192 | 9952 |
| t=7, t=14 | 1.62 | 0.0903 | 9947 |

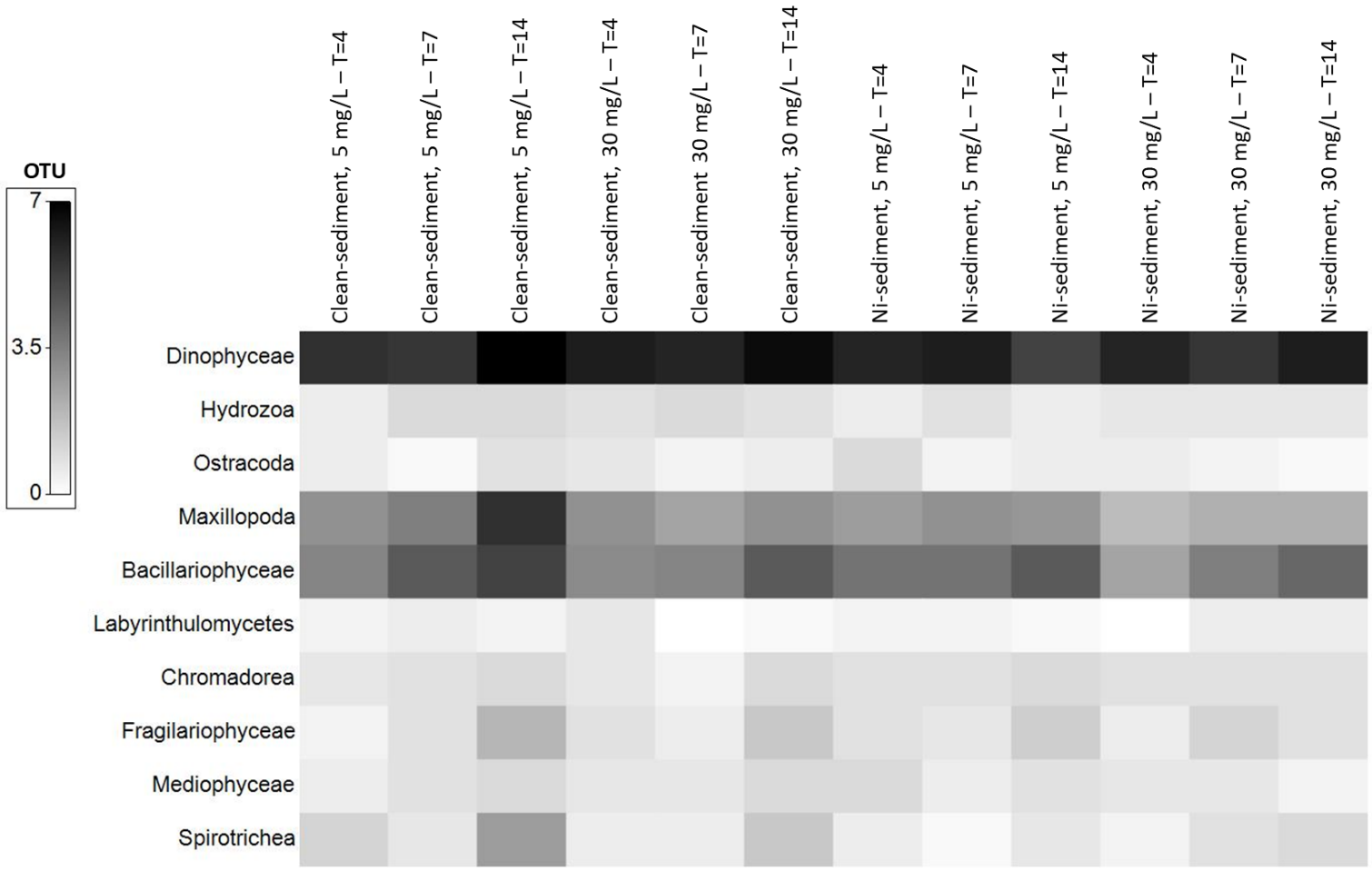



Figure 7.4. Relative richness of OTUs attributed to eukaryote taxa of the coral microbiome following exposure to clean- and Ni-sediment. Shading is indicative of the number of OTUs for each taxa present in the samples. Note, only the top ten taxa are shown here.

There was a change in the prokaryote community composition of the coral microbiome following exposure to clean- and Ni-sediment for 7 d, and after a 7-d recovery period (T=14) ($P<0.05$, Table 7.7, Figure 7.5). There was no significant interaction between any factors ($P>0.05$, Table 7.7).

There was no change in the bacterial community composition of the coral microbiomes exposed to either the clean or Ni-sediments at 5 mg TSS/L ($P=0.0968$, Table 7.8). A suspended sediment concentration of 30 mg TSS/L caused a significant difference in the bacterial communities of the coral microbiomes when exposed to the clean and Ni-sediments ($P=0.0001$, Table 7.8, Figure 7.5). Within the clean sediment matrix, the 5 and 30 mg TSS/L led to a different bacterial community composition ($P=0.0001$, Table 7.8, Figure 7.5). Within the Ni-sediment, the concentration of TSS had no effect on the bacterial community compositions ($P=0.0968$, Table 7.8, Figure 7.5). The composition of the bacterial community microbiomes changed over the duration of the experiment (t=4 and 7 d, during exposure, t=14 d, after recovery, $P<0.05$, Table 7.8).

Key taxa that were contributing to the differences in the bacterial community between the clean sediments at 5 and 30 mg TSS/L were Rhodobacteraceae (11% contribution), Hahellaceae (9% contribution), Flavobacteriaceae (4.5% contribution) and Saprospiraceae (4% contribution). The relative abundance of all four taxa declined in the 30 mg TSS/L clean sediment treatment compared to the 5 mg TSS/L treatment. Similar patterns were observed in the differences between the bacterial communities of coral microbiomes exposed to the clean- and Ni-sediments at 30 mg TSS/L. There was a decrease in the relative abundance of Rhodobacteraceae (10% contribution) and Hahellaceae (8.2% contribution) in the microbiome of corals exposed to 30 mg TSS/L of Ni-sediment compared to the same TSS and clean sediment. Compared to clean and Ni-sediment at 5 mg TSS/L, there was a decrease in the relative abundance of OTUs attributed to Flavobacteriaceae (3.4%) and Flammeovirgaceae (2.9%) in the clean and Ni-sediment at 30 mg TSS/L (Figure 7.6).

Table 7.7. The effect of clean- and Ni-sediment (matrix) at 5 and 30 mg TSS/L (treatment) on the bacterial (16S rDNA) community of the coral microbiome. Samples were taken on Day 4 and 7, during exposure and on Day 14, following a 7-d recovery period. Main test (PERMANOVA P = 0.05) investigating the effect and interaction of each of the experimental factors [a]

| Source | df | SS | MS | Pseudo-F | P(perm) | Unique perms |
|---|---|---|---|---|---|---|
| Matrix (sediment type) | 1 | 1169.9 | 1169.9 | 2.7584 | **0.0269** | 9928 |
| Treatment (mg TSS/L) | 1 | 2053.4 | 2053.4 | 4.8414 | **0.0001** | 9921 |
| Time (d) | 2 | 6843 | 3421.5 | 8.0983 | **0.0001** | 9919 |
| matrix x treatment | 1 | 915.45 | 915.45 | 2.1584 | 0.0532 | 9935 |
| matrix x time | 2 | 953.68 | 476.84 | 1.1286 | 0.3299 | 9918 |
| treatment x time | 2 | 1005.3 | 502.63 | 1.1897 | 0.2886 | 9928 |
| tank (matrix x treatment) | 8 | 3393.3 | 424.16 | 1.0039 | 0.4578 | 9861 |
| matrix x treatment x time | 2 | 1327.3 | 663.67 | 1.5708 | 0.113 | 9936 |
| time x tank (matrix x treatment) | 16 | 6760 | 422.5 | 0.99998 | 0.4824 | 9832 |
| Residual | 32 | 13520 | 422.51 | | | |
| Total | 67 | 37683 | | | | |

[a] df = degrees of freedom, SS = sum of squares, MS = mean squares

Table 7.8. The effect of clean- and Ni-sediment (matrix) at 5 and 30 mg TSS/L (treatment) on the bacterial (16S rDNA) community of the coral microbiome. Samples were taken on Day 4 and 7, during exposure and on Day 14, following a 7-d recovery period. Pair-wise tests (PERMANOVA P = 0.05) comparing the effect of sediment type, concentration of TSS and time on the community composition of the coral microbiome.

| Within level of treatment | t | P(perm) | Unique perms |
|---|---|---|---|
| 5 mg TSS/L | | | |
| Clean sediment, Ni-sediment | 1.2 | 0.0968 | 60 |
| 30 mg TSS/L | | | |
| Clean sediment, Ni-sediment | 2 | **0.0001** | 180 |
| Within level of matirx | | | |
| Clean sediment | | | |
| 5 and 30 mg TSS/L | 2.1 | **0.0001** | 60 |
| Ni-sediment | | | |
| 5 and 30 mg TSS/L | 1.63 | 0.0968 | 90 |
| Within level of time (d) | | | |
| t=4, t=7 | 2.35 | **0.0005** | 9943 |
| t=4, t=14 | 3.26 | **0.0001** | 9933 |
| t=7, t=14 | 3.06 | **0.0001** | 9932 |

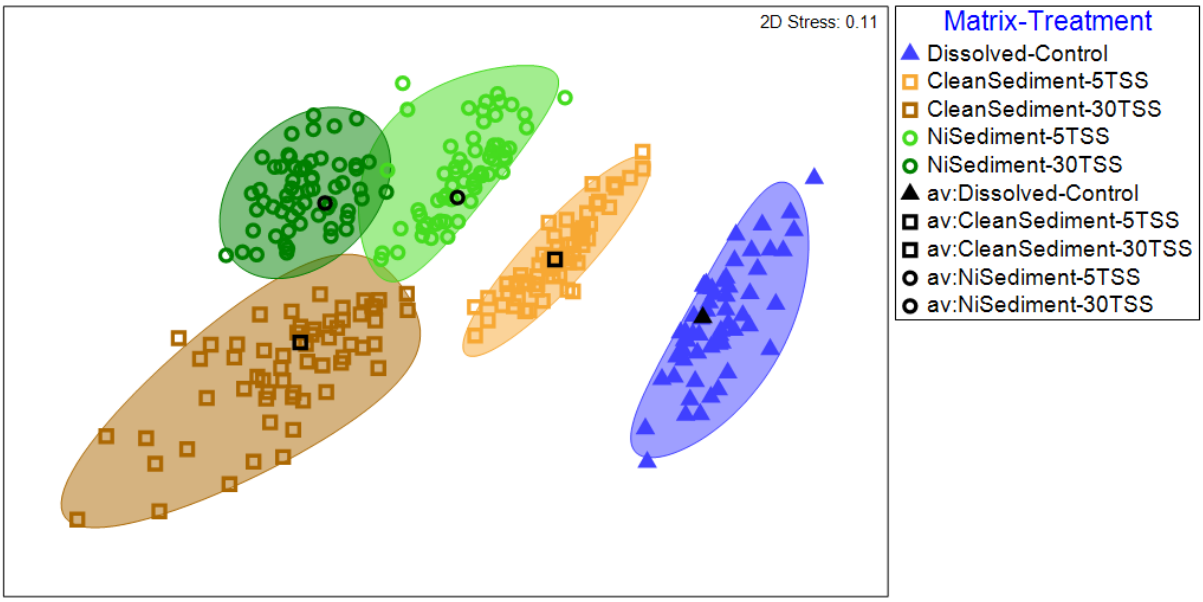



Figure 7.5. Non-metric dimensional scaling plot showing the relative similarity of the prokaryote (16S) community composition of coral microbiomes exposed to clean- and Ni-sediment at 5 and 30 mg/L TSS, relative to the control, corals exposed to natural filtered seawater only. The plot displays the average for each treatment (black shape) and bootstrap averages for each treatment (coloured symbols) and the 95% confidence around each grouping (coloured, shaded region around the mean).

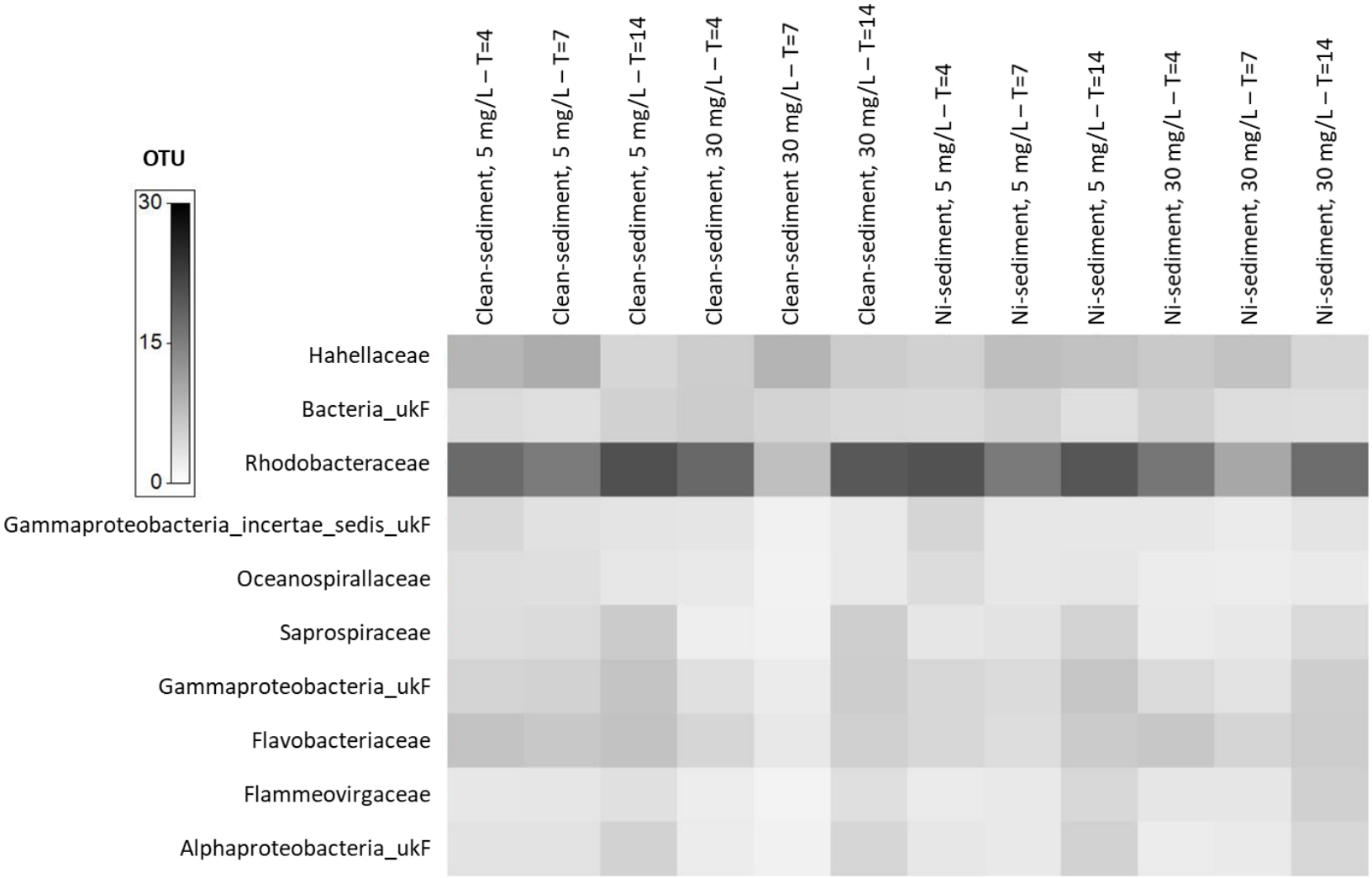



Figure 7.6. Relative abundance of OTUs attributed to prokaryote taxa of the coral microbiome following exposure to clean- and Ni-sediment. Shading is indicative of the number of OTUs for each taxa present in the samples. Note, only the top ten taxa are shown here.

## 7.4. Discussion

### *Changes in the coral microbiome community structure in response to aquaria conditions*

During the experimental period, there were significant changes in the coral microbiome over time, irrespective of the exposure (i.e. dissolved nickel or sediments). This suggests that aquaria conditions may be influencing the microbiome community structure. The increase in certain algae in the microbiome of aquaria- and laboratory-acclimatised corals could be a reflection of the diet provided to the corals. Likewise, the change in the bacterial community composition would be reflective of the bacteria present in the aquaria seawater. There was no reason to suggest that corals under aquaria and laboratory conditions were stressed as no other stress responses were observed (e.g. bleaching). However, it is important to consider how the microbiome community structure changes from field to laboratory conditions, and this is something rarely reported in the literature.

### *Changes in the coral microbiome community structure in response to suspended sediments and nickel*

The community structure of the coral microbiome remained the same following exposure to dissolved nickel (200 and 400 µg Ni/L), low TSS levels of 5 mg/L clean sediment and the field sediment (5 and 30 mg TSS/L). However, there were differences in the microbiome of corals exposed to clean- and Ni-sediment treatments at elevated TSS concentrations of 30 mg/L. The concentration of dissolved nickel in the Ni-sediment at 30 mg TSS/L treatment reached 280 µg Ni/L, within the range of the dissolved nickel treatments. In the field-sediment treatments, the dissolved nickel concentration was 1.4 ± 0.4 µg Ni/L. This reflects the difference between weakly bound nickel in the laboratory spiked Ni-sediment compared to the field-sediment where nickel was more strongly associated with the sediment particle matrix and not freely bioavailable. Overall, the results suggest that the combined effect of increased TSS and nickel exposure have significant impacts on the coral *A. muricata* and its microbiome. Similar to the results presented here, in Chapter 6 it was demonstrated that

nickel concentrations of 40, and 90 µg Ni/L caused no changes in the structure of the prokaryote community, while at 470 µg Ni/L, there were slight changes in the eukaryote community structure in the microbiome of *A. muricata* following a 4-d exposure. Here and in Chapter 6, there was a decrease in the relative abundance of the known beneficial bacteria, Hahellaceae (Neave et al., 2016). In this chapter, results showed that the relative abundance of OTUs associated with Hahellaceae decreased slightly in corals exposed to elevated concentrations of Ni-sediment (30 mg TSS/L) compared to other sediment treatments. There was a decrease in the relative abundance of Flavobacteriaceae and Flammeovirgaceae in the microbiome of corals exposed to Ni-sediment at 30 mg TSS/L. In past studies, Flavobacteriales have been identified as potentially pathogenic or opportunistic taxa in stressed corals, however, Flavobacteriaceae and Flammeovirgaceae may have a role in nitrogen fixation. It is believed that nitrogen fixation and regulation by coral microbiome organisms may facilitate coral tolerance to stress (McDevitt-Irwin et al., 2017).

There are limited laboratory-based studies which have investigated the combined effects of sediments and metal contaminants on corals and their microbiome. Bessell-Browne et al. (2017) exposed *Acropora millepora* to fine carbonate sediments (<140 µm) for 28 d and demonstrated that light attenuation associated with turbidity caused significantly higher mortality rates in corals than turbidity alone. No effects were observed in any of the suspended sediment concentrations, up to 100 mg/L, when applied alone for 28 d (Bessell-Browne et al., 2017). Berry et al. (2016) exposed *A. millepora* to suspended coal particles (<63 µm) for 28 d and found significantly different results with 10% mortality in corals exposed to 34 mg coal/L. This could be a result of a greater adhesion of the fine coal particles to the coral tissue surface which could have caused anoxia. Metal contaminants associated with coal material were low and not believed to have impacted corals during exposure (Berry et al., 2016).

Field studies have identified significant impacts of sediments and turbidity on corals and their microbiomes. Haywood et al. (2016) monitored coral cover surrounding a waste rock

disposal site around a gold mine in Papua New Guniea and observed a significant decrease in coral cover and species richness at sites closer to the disposal site. Chronic exposure to increased suspended sediment loads as a result of dredging activities significantly increased the prevalence of pathogens linked to disease in the microbiomes of nearby corals (Pollock et al., 2014). Kegler et al. (2017) also reported an increase of potentially pathogenic bacteria in coral mucus in near-shore reefs subjected to coastal eutrophication in Indonesia. In field studies such as these, there is the potential for multiple stressors to occur through the physical impact of suspended sediments and exposure to contaminants including metals and nutrients.

The limitation of laboratory-based mescosom studies is that it is not possible to consider the complex interaction of multiple stressors at one time. There is also a limit in the number of experimental replicates and this can reduce the statistical power of the results. However, the results presented in this study demonstrated that environmentally-relevant concentrations of suspended sediments, with and without nickel, during a short-term exposure, has the potential to alter the structure of the coral microbiome. This is an important consideration in the context of mining activities where there is undoubtedly an interplay between increased suspended sediments containing metals.

## 7.5. Conclusion

The results presented in this chapter demonstrate that the combined effect of elevated suspended sediments and nickel exposures has the potential to alter the structure and function of the coral microbiome. The exposure scenarios in this study were environmentally-relevant as these concentrations of nickel and total suspended sediments have been reported in SEAM. These impacts are likely to occur in the environment where corals are exposed to metal-contaminated sediments.

Unravelling the complex relationship between corals and their microbiomes is a relatively new research area. This study presents a new understanding of how the coral microbiome responds when exposed to anthropogenic stressors including increased suspended

sediments and nickel. Future work should investigate how the coral microbiome responds to such stressors in the environment and determine if such changes in the microbiome are indicative of deleterious impacts or an attempt to respond to and deal with changes in environmental conditions. Future work could also aim to identify microbial indicators of coral microbiomes that could be used as diagnostic tools for assessing changes in water quality and stress in corals.

# 8. GENERAL DISCUSSION AND CONCLUSIONS

The research presented here is part of a larger project to develop appropriate assessment tools to ensure adequate management of risks associated with nickel production in SEAM. Biological effects data for tropical ecosystems were required to assess risks of contaminant exposure and to derive WQGs to manage these risks. The SEAM region contains the world's largest deposits of nickel lateritic ores. Environmental impacts will occur if mining operations are not adequately managed. Currently, risk assessment tools and WQGs for the tropics are limited due to the sparse research on how contaminants impact tropical biota.

At the beginning of this project, a review of the published scientific literature revealed that there were limited high quality chronic nickel toxicity data for tropical marine species, and even fewer for those relevant to SEAM. Of the data available, the most sensitive SEAM species to nickel were a sea urchin, copepod and anemone. There was a significant lack of high-quality chronic data for several ecologically important taxonomic groups including cnidarians, molluscs, crustaceans, echinoderms, macroalgae and fish. No high-quality chronic nickel toxicity data were available for estuarine waters or marine and estuarine sediments. The very sparse toxicity data for tropical species limited our ability to conduct robust ecological risk assessment and required additional data generation or supplementation from other databases (e.g. temperate species) to fill data gaps.

The overall aim of this project was to address these knowledge gaps and gain an improved understanding of the environmental effects of waterborne exposure to nickel in the tropical marine ecosystems of the SEAM region. Given the crucial role that microalgae play in tropical marine environments, it is important to ensure this trophic level is represented in WQG development. This research determined the toxicity of nickel to three species of tropical marine microalgae: *Ceratoneis closterium*, from the class Bacillariophyceae, *Tisochrysis lutea* from the class Coccolithophyceae and *Symbiodinium* sp Freud Clade C from the class Dinophyceae. Based on NOEC values, *T. lutea* was the most sensitive to

nickel with a NOEC of 254 µg Ni/L, followed by *Symbiodinium* (NOEC 313 µg Ni/L) and *C. closterium* (NOEC 1606-3966 µg Ni/L).

To address the data gaps for crustaceans and molluscs (gastropods), the toxicity of nickel to three tropical marine invertebrates, the gastropod *Nassarius dorsatus*, the barnacle *Amphibalanus amphitrite*, and the copepod *Acartia sinjiensis* was investigated. All of these toxicity tests used chronic endpoints, namely larval growth, metamorphosis (transition from nauplii to cyprid larvae) and larval development for the snail, barnacle and copepod, respectively. The copepod was the most sensitive species to nickel, with development inhibited by 10% (EC10) at 5.5 (5.0-6.0) µg Ni/L (95% confidence limits (CL)). Based on EC10 values, the gastropod and barnacle showed similar sensitivities to nickel, with growth and metamorphosis inhibited by 10% at 64 (37-91) µg Ni/L and 67 (53-80) µg Ni/L, respectively. The copepod, *A. sinjiensis* is the most sensitive tropical marine species to nickel so far reported in the literature.

High quality data for corals were limited in the compilation of tropical marine nickel toxicity data (Chapter 2). This was identified as a significant data gap because scleractinian corals create the complex structural reef habitats that support many other marine species. To address this gap, the toxicity of nickel to fertilisation success in three species of scleractinian corals, *Acropora aspera*, *Acropora digitifera* and *Platygyra daedalea,* was examined. *Acropora aspera* was the most sensitive species to nickel (NOEC <280 µg Ni/L), followed by *A. digitifera* with an EC10 of 2000 µg Ni/L and *P. daedalea* (EC10 >4610 µg Ni/L).

To build on the toxicity data generated for corals, the effects of nickel on the adult life-stage of corals were investigated. Based on the growing research around the coral holobiont, that is the coral animal host and its associated microbiota, the effects of exposure to metals on the structure of the coral microbiome were studied. The common branching coral *Acropora muricata* was used to investigate the effects of dissolved nickel on corals and the associated microbiota. High concentrations of nickel caused bleaching and slight changes to the

eukaryote community structure, but no significant changes in the composition of the bacterial microbiome communities were observed.

For the final component of this research, the effects of dissolved nickel and nickel-contaminated sediments on adult corals and their microbiome were examined, using the same species (*A. muricata*) with the aim to tease apart the physical effects (sediment exposure) from the chemical effects (i.e. nickel exposure). The experiment utilised environmentally-relevant concentrations of suspended sediments and corals were exposed for 7 days to simulate a short-term pulse exposure which is likely to be encountered in the tropics. The results demonstrated that elevated suspended sediments and nickel have the potential to alter the structure of the coral microbiome. This is an important consideration in the context of mining activities where there is undoubtedly an interplay between increased suspended sediments and metals.

### 8.1. Compilation of chronic nickel toxicity data

This thesis clearly demonstrated that the sensitivity of marine species to nickel varies greatly. There is no clear pattern in sensitivity based on the geographical location of species, i.e. temperate versus tropical. In Australia, because of the limited test data on endemic species available for the derivation of WQGs, greater emphasis is placed on maximising the number of species and taxa for which toxicity data are available, rather than to prioritise data for endemic species (Warne et al., 2014, 2018). In contrast, the USEPA stipulates that all toxicity data used to derive WQG values should be for species that live and breed in North America (USEPA, 2007). Canada requires that non-indigenous species can be used to derive WQGs if it can be demonstrated that they are an appropriate surrogate species and if the exposure conditions, under which the toxicity data were derived, are relevant to Canadian waters (CCME, 2007).

Prior to this research, only six chronic toxicity data had been reported for nickel exposure to tropical marine species, half of which represented microalgae and cyanobacteria (Chapter 2). With the addition of the toxicity data for microalgae, including a dinoflagellate, a

gastropod, crustaceans and corals presented throughout this thesis, there are now sufficient tropical data to derive an interim marine guideline value that is specific for tropical waters. Having sufficient data for endemic species is frequently not the case and in these instances supplementation with the abundant temperate data is frequently used to fill data gaps (Merrington et al., 2014), but in these cases the derived guideline values are not truly region-specific.

The full tropical dataset on the biological effects of nickel to 14 species comprising nine taxonomic groups (based on Phyla) is shown in Table 8.1. For the coral, *Acropora aspera*, the endpoint was a NOEC value of <280 µg/L and there were insufficient points on the concentration-response curve to derive an EC10 (Chapter 5) so this data was omitted from the SSD. The NOEC value for the polychaete, *Hydriodes elegans,* was derived by applying a factor of 5 to a chronic EC50 value (ANZECC/ARMCANZ, 2000; Warne et al., 2018). Guideline values of greater reliability can be derived without the inclusion of converted data (Warne et al., 2018). There was no difference in the derived Protective Concentration (PC) for protecting 95% (PC95) and 99% (PC99) of species (Table 8.2) with or without the polychaete data included in the SSD. However, the aim of this study was to compile data and derive a region-specific WQG for SEAM, therefore, the polychaete data was omitted from the SSD and this resulted in a total of 11 species representing eight taxonomic groups (Figure 8.1).

The derived tropical marine PC values for different levels of ecosystem protection are shown in Table 8.2 (derived based on ANZECC/ARMCANZ, 2000 and Warne et al., 2018). It is the 95% species protective concentration value (PC95) of 7.4 µg Ni/L that would be mostly applied in slightly-to-moderately disturbed systems (Figure 8.1, Table 8.2). This value is above the typically reported background concentrations of nickel in seawater (<5 µg Ni/L, Apte et al., 2006, DeForest and Schlekat, 2012). The Australian and New Zealand PC95 value for nickel (based on temperate marine data) is 70 µg Ni/L. Because this was insufficiently protective of some species, the default guideline for slightly to moderately

disturbed systems was set at 7 µg Ni/L (99% species protection) (ANZECC/ARMCANZ, 2000). The USEPA derived a chronic nickel guideline for saltwater of 8.2 µg Ni/L, similar to the ANZECC/ARMCANZ guideline value. The European Union Environmental Risk Assessment of Nickel derived an HC5 (= PC95) value of 17.2 µg Ni/L for temperate marine waters (Nickel Institute, 2012). DeForest and Schlekat (2012) undertook further toxicity testing with temperate marine species and provided an additional two data to derive the HC5. In their study, the most sensitive species to nickel was actually a tropical species of a long-spined sea urchin (*Diadema antillarum*) from the Caribbean region, which had an EC10 of 2.9 µg Ni/L. However, this toxicity test was carried out at 20˚C, so was not included in the tropical compilation presented in Chapter 2. DeForest and Schlekat (2012) derived a marine nickel HC5 of 3.9 µg Ni/L (including this tropical sea urchin) and 21 µg Ni/L (when the sea urchin data were excluded due to lack of relevance to European marine waters). This value is similar to that originally derived by the Nickel Institute in 2012. The PC95 value of 7.4 µg Ni/L derived in this thesis is within the range of values derived for nickel in temperate marine waters by DeForest and Schlekat (2012), the USEPA and ANZECC/ARMCANZ. This suggests that temperate and tropical marine species have similar sensitivities to nickel, based on chronic data.

Warne et al. (2018) provided guidance for assessing the reliability of PC values derived from SSD methods. This is based on the sample size (number of species for which toxicity data are available), the type of data (chronic, chronic and acute, or converted values), and by visual assessment of the fit of the SSD to the toxicity data (i.e. good or poor). In the SSD shown in Figure 8.1, there are a total of 11 chronic data and the fit of the SSD to the toxicity data was poor, placing the classification of this PC95 value as moderate reliability. It should be noted that this is not a regulatory value, but a reflection of the continued and ongoing research to produce high quality nickel toxicity data for tropical marine waters. The interim guideline values derived in this thesis (Table 8.2.) will be validated with field testing to

investigate exposure concentrations of nickel in marine environments of SEAM and to assess community responses to increasing nickel concentrations.

There are inherent uncertainties that should be considered when applying guideline values, for example, the difference between laboratory waters and natural waters, and the absence of insensitive taxa such as fish from the SSD. In this instance, it is also important to note that the SSD and derived PC95 value for nickel is largely driven by the copepod data, the most sensitive species, and this is a relatively new endpoint when evaluating ecological relevance. Guideline values ultimately provide guidance and where uncertainties arise, additional lines of evidence should be sought.

Table 8.1. Compilation of toxicity estimates for the biological effect of nickel on a range of tropical marine organisms, summarised from Chapter 2, including existing data and data generated in this thesis[a]

| Phylum | Common name used in SSD | Scientific Name | Endpoint | Temperature (°C) | Toxicity Measure | Reported toxicity value (µg/L) | Toxicity value used in SSD (µg/L) | Reference |
|---|---|---|---|---|---|---|---|---|
| Cyanobacteria | Cyanobacteria | *Cyanobacteria Cyanobium* sp. | inhibition in growth rate | 25 | EC10 | 3700 | 3700 | Alqueza and Anastasi (2013) |
| Bacillariophyta | Microalgae | *Ceratoneis closterium*[b] | inhibition in growth rate | 27 | EC10 | 2870 | 2870 | This study Chapter 3 |
| Haptophyta | Microalgae | *Tisochrysis lutea*[c] | inhibition in growth rate | 27 | EC10 | 330 | 330 | This study Chapter 3 |
| Miozoa | Symbiodinium | *Symbiodinium* sp Freud Clade C | inhibition in growth rate | 27 | NOEC | 310 | 310 | This study Chapter 3 |
| Echinodermata | Sea urchin | *Diadema savignyi* | fertilisation and development | 25 | NOEC | 23.5 | 24 | Rosen et al. (2015) |
| Annelida | Polycheate | *Hydriodes elegans*[d] | larval settlement | 28 | EC50 | 162 | 32 | Gopalakrishnan et al. (2008) |
| Cnidaria | Anemone | *Aiptasia pulchella* | reproduction-total number juveniles | 25 | EC10 | 66 | 66 | Howe et al. (2014) |
| Cnidaria | Coral | *Platygyra daedalea* | fertilisation | 25 | NOEC | 920 | 920 | This study Chapter 5 |
| Cnidaria | Coral | *Acropora digitifera* | fertilisation | 25 | EC10 | 2000 | 2000 | This study Chapter 5 |
| Cnidaria | Coral | *Acropora aspera* | fertilisation | 25 | NOEC | <280 | NA | This study Chapter 5 |
| Mollusca | Snail | *Nassarius dorsatus* | juvenile growth | 29 | EC10 | 64 | 64 | This study Chapter 4 |
| Crustaceae | Barnacle | *Amphibalanus amphritrite* | nauplii metamorphosis | 29 | EC10 | 67 | 67 | This study Chapter 4 |
| Crustaceae | Copepod | *Acartia sinjiensis* | larval development | 30 | EC10 | 5.5 | 5.5 | This study Chapter 4 |

EC10 = 10% effect concentration. NOEC = No observable effect concentration.
[a] This table includes measured nickel data which passed the quality assurance criteria established in Gissi et al. (2016), ANZECC/ARMCANZ (2000), and Warne et al. (2015).
[b] Previously known as *Nitzcshia closterium*, geometric mean taken from two EC10 values
[c] Previously known as *Isochrysis* sp.
[d] Chronic EC50 converted to NOEC value, divide by 5 (Warne et al., 2018)
[e] Acute value, not included in SSD
NA = not applicable, toxicity value not used in SSD

Table 8.2. Protective concentration (PC) values for nickel derived using the species sensitivity distribution shown in Figure 8.1 for SEAM species (SSD for tropical dataset not shown), for different levels of protection based on Warne et al. (2018)

| | PC (±95% CL) | |
|---|---|---|
| **Level of protection** | **SEAM** | **Tropical[a]** |
| PC90 – 90% species protection for disturbed systems | 17 (8.2 – 78) | 16 (8.4 – 54) |
| PC95 – 95% species protection for slightly-to-moderately disturbed systems | 7.4 (1.8 – 35) | 8.6 (2.5 – 30) |
| PC99 – 99% species protection for pristine systems | 1.3 (0.1 – 27) | 2.8 (0.1 – 12) |

[a] Includes toxicity data for the Polycheate, not found in SEAM

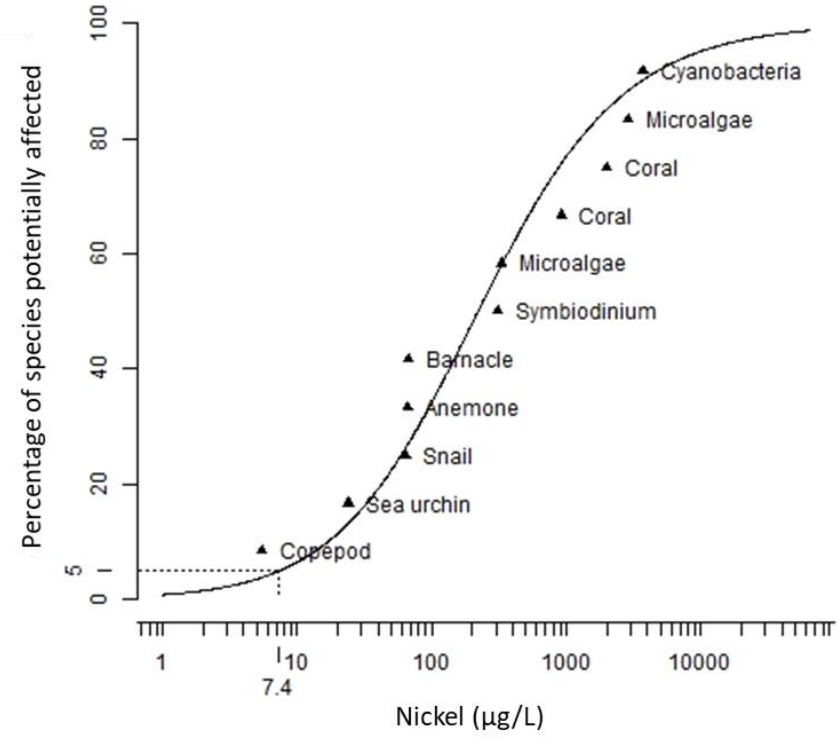



Figure 8.1. Species sensitivity distribution using chronic, measured nickel toxicity data, for data that passed the quality assurance criteria established in Chapter 2. Data presented in this SSD only includes species which were found to be relevant to SEAM. The value indicated on the x-axis by the dotted line represents the PC95 value.

## 8.2. Compilation of chronic copper toxicity data

Throughout this research copper was used as a reference toxicant to allow for comparisons with previous studies; however, copper is a recognised contaminant in marine ecosystems shown to have deleterious effects to many organisms (Lawes et al., 2016). While there is substantially more copper data available for tropical species, this dataset is still limited compared to temperate datasets. Therefore, those data presented in this thesis will also contribute to the development of a WQG for copper in tropical waters.

Overall, copper was more toxic than nickel to all species tested. Sensitivity to copper among the species tested in this study varied, between 0.97 – 13 µg Cu/L, based on the 10% effect concentration values. For microalgae, growth rates were inhibited by 10% at 0.87-3.3 µg Cu/L. *C. closterium* was the most sensitive species, followed by *T. lutea* and *Symbiodinium* sp. In Chapter 4, the copepod, *A. sinjiensis* was more sensitive than the snail and barnacle with an EC10 value of 1.4 µg Cu/L, followed by 3.7 and 10 µg Cu/L for the snail and barnacle, respectively. With respect to coral fertilisation, *A. aspera* was the most sensitive species to copper, with an EC10 of 5.8 µg Cu/L. The EC10 for *P. daedalea* was 16 µg Cu/L, similar to previous studies (Chapter 5). In Chapter 6 it was shown that exposure to copper not only resulted in bleaching, but also altered the composition of both the eukaryote and bacterial communities of the corals' microbiome.

From the SSD presented in Figure 8.2, the derived PC95 value was 1 µg Cu/L (Table 8.3). This is similar to the guideline value derived for temperate marine waters in Australasia and the US of 1.3 µg/L (ANZECC/ARMCANZ, 2000; USEPA, 2016)

Table 8.3. Protective concentration (PC) values for copper derived using the species sensitivity distribution shown in Figure 8.2, for different levels of protection based on Warne et al. (2018)

| **Level of protection** | **PC (±95% CL)** |
|---|---|
| PC90 – 90% species protection for disturbed systems | 1.2 (0.8-2.5) |
| PC95 – 95% species protection for slightly-to-moderately disturbed systems | 1 (0.73-2.3) |
| PC99 – 99% species protection for pristine systems | 0.74 (0.52-2.0) |

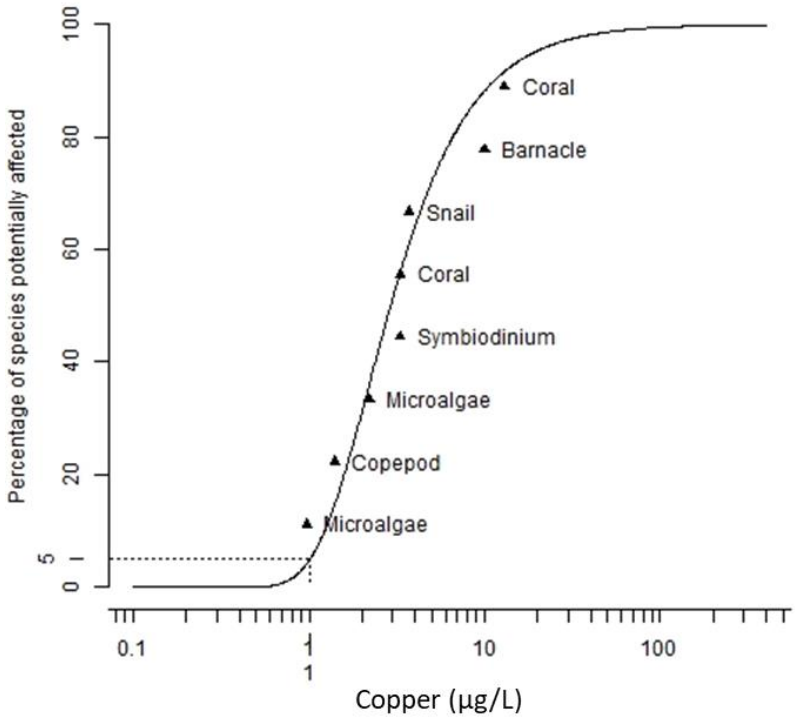



Figure 8.2. Species sensitivity distribution using chronic, measured copper tropical toxicity data generated throughout this thesis. The value indicated on the x-axis by the dotted line represents the PC95 value.

## 8.3. Conclusions and recommendations for future research

A range of different test protocols were implemented in this study, some better established than others. Toxicity tests with microalgae are well established and easily reproducible and there is substantially more data available in the literature with which to compare results. The toxicity test protocol for the Dinoflagellate, *Symbiodinium* was developed in this study, and future work could develop this protocol further, for example, by removing culture media altogether, or optimising nutrient concentrations to improve growth rates in culture and tests. Other strains of *Symbiodinium*, isolated from different eukaryote hosts are also available for test development.

Toxicity tests with the snail and barnacle are robust and these protocols have been used to investigate the toxicity of several metals (including copper, aluminium, gallium and molybdenum) to these species (Trenfield et al., 2016; van Dam et al., 2016). The toxicity test protocol with the copepod was developed during this research and while it is a highly relevant test endpoint and the data acquired so far for copper and nickel is reproducible, it is a relatively labour-intensive protocol and so improvements could be made to make this test more applicable for routine toxicity assessment. The use of scanning and image recognition technology also has the potential to provide more biological information on the effects of contaminants on larval development tests for copepods and other invertebrate species (Binet et al., 2019).

As discussed in Chapter 5, Section 5.4.4, there is inherent variability in the sensitivity of coral gametes to metals due to natural phenotypic variability and the environmental conditions that the parent colonies are found in prior to testing. This is to be expected when working with organisms directly from the field, unlike organisms cultured in the laboratory which may display less phenotypic variability and therefore show less variation in their sensitivities to metals. However, test organisms taken directly from the environment could provide a better representation of wild populations. There are pros and cons to working with

wild and laboratory organisms, all of which should be considered when acquiring biological effects data for water quality guideline development.

Single-species laboratory data are valuable in deriving WQG, however, there is uncertainty in applying such data to the real world to predict effects in the field. Future work should also consider ecological assessments of communities adjacent to mining activities and investigate the impacts, if any, occurring on these communities. This would provide multiple lines of evidence and further support WQG and risk assessment tools. Access to field sites during this research program was attempted, however it was difficult due to safety and liability issues. As such, field work could not be achieved within the time constraints of this project.

It is noted that the experiments in this study investigated a single metal (nickel or copper) exposure, and this is rarely the case in the environment. However, such data are required for water quality guideline development and they also provide a basic understanding of the responses of certain organisms to individual metals. Future work should investigate multiple stressor effects including multiple metal contaminants (e.g. nickel and cobalt), anions (e.g. sulfates) as well as environmental stressors resulting from climate change impacts (e.g. increased sea surface temperature and ocean acidification). In tropical environments, periods of high rainfall may lead to an increase in sediment loads (potentially containing metal contaminants) in coastal systems. There should also be consideration around the different types of exposures, dissolved verses particulate, and the effects that increased suspended sediment associated with mining activities can have on adjacent tropical marine ecosystems.

The region of SEAM exhibits a unique geochemistry composed of lateritic soils rich in mafic minerals including magnesium, iron and nickel (Van der Ent et al., 2013). Topsoils are characterised by low pH, resulting in low pH freshwaters and sediments are often high in metal oxides including iron, manganese and aluminium which can bind nickel and reduce bioavailability and toxicity (Costello et al., 2016). These characteristics strongly influence the

bioavailability and fate of nickel in tropical ecosystems (Van der Ent et al., 2013), however; there is a limited toxicity data on lateritic-based ecosystems of tropical SEAM. Future research should address nickel bioavailability and toxicity from sediments within SEAM to a range of estuarine and marine organisms.

A large component of this thesis focused on the effects of nickel on corals. This was directed by the key role coral reefs play in tropical marine environments and due to a significant gap in the literature for toxicity data but also in understanding how the coral holobiont responds to metal stress. Unravelling the complex relationship between corals and their microbiomes is a relatively new research area. The results showed that bleaching and alterations to the microbiome were evident at relatively high metal concentrations, only observed in highly polluted environments. Collectively, metal and suspended sediment exposure not only has the potential to cause bleaching in corals, but also the capacity to alter the coral microbiome which is inherently linked to coral health. The results reported in this study provide a new understanding of how the coral microbiome responds when exposed to anthropogenic stressors including increased suspended sediments and metals. Future work should investigate how the coral microbiome responds to such stressors in the environment and should also aim to identify microbial indicators of coral microbiomes that could be used as diagnostic tools for assessing changes in water quality and stress in corals (Glasl et al., 2017). Such tools would be invaluable in identifying impacts before deleterious effects are observed such as bleaching or mortality. In addition, it would be beneficial to investigate changes in coral microbiomes and coral health in response to lower, environmentally relevant concentrations of dissolved metals; for example, concentrations of <10 µg/L for nickel and copper, as these are levels which have been reported in disturbed environments (Chapter 6, section 6.4.1.). It would also be beneficial to repeat these experiments over a short-term (4-7 days) and a long-term (4-8 weeks) exposure period to determine the resilience of the coral microbiome to deal with metal stress.

In this study ecogenomics or DNA metabarcoding techniques were used to assess changes in the coral microbiome when exposed to metals under controlled laboratory conditions. The same techniques could also be applied to real communities in the environment, in both benthic and pelagic ecosystems to understand the impacts of contaminants in the environment and the influence of such stressors on community structure and function. Changes in community structure have been used in preliminary studies in benthic systems to assess the biodiversity of estuarine and coastal environments and to develop site-specific water quality guidelines (Aylagas et al., 2016; Yang et al., 2017). However, technologies and methods around DNA-metabarcoding are continuing to evolve and currently there are limited standardized methods for the sampling, extraction, sequencing and analysis of environmental DNA from field samples, therefore making comparisons across multiple studies difficult. While these tools are useful, they are still being developed and such information on ecological diversity should be used as one of many multiple lines of evidence.

The development of ecologically-relevant risk assessment tools for nickel in tropical marine environments is hindered due to the paucity of data on the biological effects of nickel on tropical species. Through this research, results on the toxicity of nickel to three species of microalgae, one gastropod, two crustaceans and four different species of coral (early life stages and adult), relevant to tropical Asia-Pacific have been presented. These test organisms were ideal species to include in the SSD for nickel due to their sensitivity to the metal, in particular the copepod. With the inclusion of key tropical species, and high-quality chronic toxicity data, greater reliability can be placed on the PC95 value reported in this study. It is anticipated that the data presented here will contribute to the development of an ecologically-relevant water quality guideline for nickel in tropical marine waters of SEAM. Given the potential concentrations of nickel in the environment in close surrounds to nickel mines and facilities, there is potential risk of nickel toxicity to tropical marine organisms in these locations.

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# APPENDICES

## Appendix A. Toxicity Tests with Tropical Marine Biota and Gap Analysis of Nickel Toxicity Data

Table A1. Summary of tropical marine toxicity tests with cyanobacteria, microalgae, macroalgae and seagrass. Light blue shading identifies SEAM relevant, available test species; dark blue shading signifies SEAM relevant test species which are unlikely to be available; no shading means not relevant to SEAM.

| Species | Growth phase/initial cell density | Endpoint | Acute or Chronic? | Test duration | Temp (°C) | Salinity (‰) | pH | Reference |
|---|---|---|---|---|---|---|---|---|
| **Cyanobacteria** | | | | | | | | |
| *Cyanobacteria Cyanobium sp.* | 6 x 10^3 cells/mL | growth rate | chronic | 72 h | 25 | 33 | 8 | Alquezar and Anastasi (2013) |
| **Mircoalgae** | | | | | | | | |
| *Isochrysis galbana* | log-phase. 10^3 - 10^4 cells/mL | growth rate | acute | 72 h | 24 | NR | NR | Moreno-Garrido et al. (2000) |
| | log-phase. 6.6 x 10^4 cells/mL | | | | 20 | NR | 8 | Debelius et al. (2009) |
| *Nitzschia closterium* [a] | log-phase. 3-5 x 10^4 cells/mL | growth rate | chronic | 72 h | 27 | NR | NR | Florence et al. (1994) |
| *Odontella mobiliensis* | 4-5 d log-phase, 1.8 ± 0.23 x 10^4 cells/mL | growth rate, cell morphology, size, nitrate reductase, antioxidant enzyme activity | chronic | 72 h - 7 d | 25 ± 1 | 30 | 8.0 ± 0.3 | Manimaran et al. (2012) |
| *Pyrocystis lunula (dinoflagellate)* | x10^5 cells/mL | bioluminescence | acute | 24 h | 25 ± 1 | 33 ± 0.5 | 8.1-8.4 | Bao et al. (2011) |
| *Navicula sp.* | log-phase, 3 x10^4 cells/mL | growth rate, inhibition of photosynthetic efficiency and pigment concentrations | chronic | 72 h | NR | NR | NR | Magnusson et al. (2008) |
| | log-phase, 1.5 x10^5 cells/mL | photosynthetic efficiency | acute | 4 h | | | | Magnusson et al. (2010) |
| *Nephroselmis pyriformis* | log-phase, 3 x10^4 cells/mL | growth rate, inhibition of photosynthetic efficiency and pigment concentrations | chronic | 72 h | NR | NR | NR | Magnusson et al. (2008) |
| | log-phase, 1.5 x10^5 | photosynthetic efficiency | acute | 4 h | | | | Magnusson et al. (2008) |
| *Cylindrotheca closterium* | log-phase, 4 x10^5 | photosynthetic efficiency | acute | 4 h | NR | NR | NR | Magnusson et al. (2008) |
| *Chaetoceros gracilis* | NR | growth rate inhibition | chronic | 72 h | 25 | 30 | 7.5 | Zhen Wang. Pers comm. |
| *Skeletonema costatum* | NR | growth rate inhibition | chronic | 72 h | 20 | NR | NR | Onduka et al. (2013) |
| **Plantae** | | | | | | | | |
| **Macroalgae** | | | | | | | | |
| *Ulva reticulata* | NR | growth rate | chronic | 7 d | 25 | 20 - 40 | NR | Mamboya et al. (2009) |
| *Ruppia maritima* | leaves and roots, 5 cm | growth and chlorophyll a | chronic | 7 d | 25 | 12 | NR | Castro et al. (2015) |
| **Seagrass** | | | | | | | | |
| *Cymodocea serrulata* | NR | photosynthesis | acute | 5 d | 20-35 | 32-39 | 8.1-8.4 | Haynes et al. (2000) |
| *Halophila ovalis* | NR | photosynthesis | acute | 5 d | 20-35 | 32-39 | 8.1-8.4 | |
| *Zostera capricorni* | NR | photosynthesis | acute | 5 d | 20-35 | 32-39 | 8.1-8.4 | |
| *Halophila ovalis* | Single leaves | photosynthesis | acute | 24 h | 26 | 34 - 36 | NR | Wilkinson et al. (2015) |

[a] Now known as *Ceratoneis closterium*

Table A2. Summary of tropical marine toxicity tests with rotifers and microcrustaceans. Light blue shading identifies SEAM relevant, available test species; dark blue shading signifies SEAM relevant test species which are unlikely to be available; no shading means not relevant to SEAM.

| Species | Lifestage | Endpoint | Acute or Chronic? | Test duration | Temp (°C) | Salinity (‰) | pH | Reference |
|---|---|---|---|---|---|---|---|---|
| **Rotifers** | | | | | | | | |
| *Brachionus plicatillis* | newly hatched nauplii | survival | acute | 24 h | 25 | NR | NR | Martins et al. (2007) |
| | 0-2 h nauplii | survival | acute | 48 h | 24-26 | 15 | 7.3-7.6 | Arnold et al. (2010) |
| | 0-3 h nauplii | population intrinsic growth rate | chronic | 96 h | 24-26 | 15 | 7.3-7.6 | |
| **Crustacea** | | | | | | | | |
| **Copepods** | | | | | | | | |
| *Acartia lilljeborgi* | adult | survival | acute | 48 h | 25 ± 2 | 33.5 ± 1.5 | NR | Nipper et al. (1993) |
| *Acartia pacifica* | adult | reproduction | chronic | 10 d | 25 | 25 | NR | Mohammed et al. (2010) |
| | | survival | acute | 48 h | 25 | 25 | NR | |
| *Acartia sinjiensis* | adult | survival | acute | 24 and 48 h | 30 | 35 | 8.1 ± 0.2 | Gissi et al. (2013) |
| *Gladioferens imparipes* | adult females | reproduction | chronic | 28+ d | 20-25 | 35 | 8-8.2 | Geotech. Tristan Stringer, Pers comm. |
| | nauplii 1 and 2 | development | | 5-7 d | | | | |
| *Parvocalanus crassirostris* | junvenile | survival | acute | 48 h | 27 ± 2 | | | ESA SOP 124 |
| *Temora stylifera* | adult | survival | acute | 48 h | 25 ± 2 | 33.5 ± 1.5 | NR | Nipper et al. (1993) |
| *Tigrious japonicus* | adult | survival | acute | 96 h | 24.5 ± 1.0, 34.5 ± 1.0 | 15 ± 0.5, 34.5 ± 0.5, | NR | Kwok and Leung (2005) |
| | < 24 h nauplii | complete life-cycle, development and reproduction | chronic | 20-30 d (2 broods) | 25 ± 1.0 | 30 ± 0.5 | 7.9-8 | Kwok et al. (2008) |
| | < 24 h nauplii | complete life-cycle, development and reproduction | chronic | 21 d | 25 ± 1.0 | 33 ± 0.5 | 8.1-8.4 | Bao et al. (2014) |
| | adult females | reproduction | chronic | 10 d | 20 | 25 | NR | Mohammed et al. (2010) |
| | | survival | acute | 48 h | 20 | 25 | NR | |
| *Apocyclops borneoensis* | adult females | reproduction | chronic | 10 d | 30 | 20 | NR | Mohammed et al. (2010) |
| | | survival | acute | 48 h | 30 | 20 | NR | |
| **Mysids** | | | | | | | | |
| *Mysidopsis juniae* | NR | lethality | acute | 96 h | NR | NR | NR | Figueiredo et al. (2015) |
| | juvenile and 1-d old adult | survival | acute | 96 h | 25 ± 2 | 33.5 ± 1.5 | NR | Nipper et al. (1993) |
| *Americamysis bahia* [a] | post-larval stage | growth and survival | chronic | 96 h | 25 ± 1 | 30 ± 2 | NR | Lussier et al. (1999) |
| | < 48 h old mysids | survival | acute | 48 h | 20 ± 1 | 30 | NR | Ho et al. (1999) |
| *Mysidopsis intii* | 2-d old neonates | survival | acute | 96 h | 20 | 34 | NR | Hunt et al. (2002) |
| | | life-cycle test: survival, growth, reproduction | chronic | 28 h | | | | |
| **Shrimps** | | | | | | | | |
| *Artemia urmiana (brine shrimp)* | <24 h old nauplii | growth and survival | chronic | 11-day | 27± 1 | 35 | NR | Asadpour et al. (2013) |
| | adult | bioaccumulation | acute | 24 h | 27± 1 | 75 | NR | |
| *Artemia franciscana (brine shrimp)* | <24 h old nauplii | growth and survival | chronic | 11-day | 27± 1 | 35 | NR | |
| | adult | bioaccumulation | acute | 24 h | 27± 1 | 75 | NR | |
| *Artemia salina (brine shrimp)* | <24 h old nauplii | survival | acute | 24 h | 25 | 35 | NR | Martins et al. (2007) |
| *Artemia* | embryos | emergence, development and survival | chronic | 72 h | 28 | NR | NR | MacRae and Pandey (1991) |
| **Seed shrimps (Ostracods)** | | | | | | | | |
| *Cypris sp. (seed shrimp)* | 0.25 - 0.3 mm diameter | survival | acute | 24 - 96 h | 22-24.5 | 5 | NR | Oyewo and Don-Pedro (2001) |

[a] Previously known as *Mysidopsis bahia*

Table A3. Summary of tropical marine toxicity tests with macrocrustaceans. Light blue shading identifies SEAM relevant, available test species; dark blue shading signifies SEAM relevant test species which are unlikely to be available; no shading means not relevant to SEAM.

| Species | Lifestage | Endpoint | Acute or | Test duration | Temp | Salinity | pH | Reference |
|---|---|---|---|---|---|---|---|---|
| **Amphipods** | | | | | | | | |
| *Elasmopus rapax* | juvenile | survival | acute | 96 h | 25 ± 1 | 33 ± 0.5 | 8.1-8.4 | Bao et al. (2011) |
| | adult | survival | acute | 48, 96 h | 24 ± 0.5 | 30 | 8.1 ± 0.05 | Zanders and Rojas (1992) |
| | | bioaccumulation | chronic | 240 h | | | | |
| *Ampelisca abdita* | juvenile | survival | acute | 24 h | 20 ± 1 | 30 | NR | Ho et al. (1999) |
| **Barnacles** | | | | | | | | |
| *Amphibalanus amphitrite amphitrite* | 4-5 day old cyprids (larvae) | metamorphosis and settlement | chronic | 48h | 27 | NR | NR | Afsar et al. (2003) |
| | stage 2 nauplii | survival | acute | 24 h | 28 | NR | NR | Rittschof et al. (1992) |
| | cyprids | settlement | chronic | 22 h | 28 | NR | NR | |
| *Amphibalanus amphitrite* | ≤ 3 h old stage II larvae | larval development (inhibition of | chronic | 96 h | 29 ± 1 | 30-35 | 7.8-9 | van Dam et al., (2016) |
| *Balanus amphitrite* [a] | 4d into cyprid larval stage | mortality | acute | 24 h | 27 | 33 ± 0.5 | 8.1-8.4 | Bao et al. (2011) |
| **Crabs** | | | | | | | | |
| *Scylla serrata* | instar 2 crablets | growth and survival | chronic | 18 d | 20-35 | 0-40 | NR | Ruscoe et al. (2004) |
| | zoea stages I-V, megalopa | survival | acute | 48 h | 26.4-28.6 | 28-32 | 7.7-8.3 | Neil et al. (2005) |
| | | survival and development from | chronic | 19 d | | | | |
| *Tunicotheres moseri* | zoea I, II and megalopa | survival | acute | 96 h | 25 | 37 | NR | Greco et al. (2001) |
| *Clibanarius africanus (Hermit* | 0.48 g ex-shell | survival | acute | 24 - 96 h | 22 - 24.5 | 15 | NR | Oyewo and Don-Pedro (2001) |
| **Prawns** | | | | | | | | |
| *Penaeus merguiensis* | juvenile (~ 6 weeks old) | survival | acute | 96 h | 27 | 20 | NR | Ahsanullah and Ying (1995) |
| | | growth | chronic | 14 d | 27 | 20 | NR | |
| *Penaeus monodon* | juvenile (~ 6 weeks old) | survival | acute | 96 h | 27 | 20 | NR | |
| *Penaeus merguiensis* | juvenile | survival | acute | 96 h | 20-35 | 30 and 36 | NR | Denton and Burdon-Jones (1982) |
| *Penaeus monodon* | juvenile | growth | chronic | 14 and 30 d | 27 ± 0.5 | 32 ± 0.1 | NR | Florence et al. (1994) |
| *Penaeus merguiensis* | | survival | acute | 96 h | 20 | | | |
| *Litopenaeus vannamei* [b] | NR | survival | acute | 96 h | NR | NR | NR | Neff et al. (2000) |
| | juvenile | accumulation and regulation | chronic | 10 d | 25 | NR | NR | Nunez-Nogueira et al. (2012) |
| *Metapenaeus ensis* | 3 d postlarval (PL3) stage | survival | acute | 48 h | 27 ± 1 | NR | 8.1 | Wong et al. (1993) |
| | | feeding behaviour | | 24 h | | | | |

[a] Now known as *Amphibalanus amphiritite*

[b] previously known as *Penaeus vannamei*

Table A4. Summary of tropical marine toxicity tests with molluscs, bivalves. Light blue shading identifies SEAM relevant, available test species; dark blue shading signifies SEAM relevant test species which are unlikely to be available; no shading means not relevant to SEAM.

| Species | Lifestage | Endpoint | Acute or Chronic? | Test duration | Temp (°C) | Salinity (‰) | pH | Reference |
|---|---|---|---|---|---|---|---|---|
| **Bivalves** | | | | | | | | |
| *Crassostrea belcheri (oyster)* | adult | metabolism, oxygen consumption, ammonia excretion | acute | 12 h | 26 ± 1 | 30 ± 2 | NR | Elfwing and Tedengren (2002) |
| *Crassostrea iredalei (oyster)* | newly fertilised egg | development to larvae stage D-shaped veliger | chronic | 48 h | 25 | NR | NR | Ramachandran (1997) |
| *Crassostrea lugubris (oyster)* | adult | metabolism, oxygen consumption, ammonia excretion | acute | 12 h | 26 ± 1 | 30 ± 2 | NR | Elfwing and Tedengren (2002) |
| *Crassostrea gigas (oyster)* | embryo (newly fertilised eggs) | larval developement to normal D-shape | sub-chronic | 24 h | 24 | 35 | NR | Libralato et al. (2007) |
| *Crassostrea virginica (Eastern oyster)* | embryos | survival | acute | 48 h | 25 ± 1 | 24 ± 2 | NR | Calabrese et al. (1977) |
| | larvae | survival | acute | 12 d | | | | |
| *Pinctada maxima (oyster)* | 4 mo old (25-40 mm) | feeding behaviour | chronic | 7 d | 26-30 | NR | 8.1-8.2 | Negri et al. (2004) |
| *Saccostrea cucullata (oyster)* | adult | metabolism, oxygen consumption, ammonia excretion | acute | 12 h | 26 ± 1 | 30 ± 2 | NR | Elfwing and Tedengren (2002) |
| *Saccostrea echinata (oyster)* | adult | metal accumulation | chronic | 30 d | 20 and 30 | 36 and 20 | NR | Denton and Burdon-Jones (1981) |
| | larvae | development | chronic | 48 h | 29 ± 1 | NR | NR | ESA SOP 106 |
| *Pteria colymbus (Pearl oyster)* | embryos | development to pluteus stage | chronic | 24 h | 27 | 36.6 | 8 | Rumbold and Snedaker (1997) |
| *Tridacna maxima (Small Giant clam)* | fecondated egg | development to D larvae (veliger) | chronic | 48 h | 27 (summer) | 34 | 8.4 | Aquabiotech. Jocelyn Senia, Pers comm. |
| *Saccostrea rhizophora (Mangrove Oyster)* | fecondated egg | development to D larvae (veliger) | chronic | 48 h | 27 (summer) | 34 | 8.4 | |
| *Mimachlamys gloriosa (scallop)* | fecondated egg | development to D larvae (veliger) | chronic | 48 h | 24 (winter) | 34 | 8.4 | |
| *Bractechlamys vexillum (seashell)* | fecondated egg | development to D larvae (veliger) | chronic | 48 h | 24 (winter) | 34 | 8.4 | |
| *Garfrarium tumidum (clam)* | adult shell width ≥ 35 mm. | Ni uptake and bioaccumulation | chronic | 14 d | 25 ± 1 | 35 ± 1 | 8.0 ± 0.1 | Hédouin et al. (2007) |
| *Isognomon isognomon (oyster)* | adult shell width ≥ 70 mm. | Ni uptake and bioaccumulation | | | | | | |
| *Malleus regula (oyster)* | adult shell width ≥ 70 mm. | | | | | | | |
| *Musculus lateralis* | embryos | survival | acute | 48 h | NR | NR | NR | Lussier et al. (1999) |
| *Perna viridis (Green-lipped mussel)* | spermatozoa | sperm motility and ultrastructure | sub-chronic | 0, 30 and 60 min | 22 | 30 | NR | Au and Chiang (2000). Au and Reunov (2001) |
| *Haliotis rufescens (Abalone)* | 48 h old veliger larvae | normal shell development | acute | 48 h | 15 | 34 | NR | Hunt et al. (2002) |
| | < 1 h old fertilised embryos | larvae/juvenile metamorphosis | chronic | 14 d | | | | |
| | | juvenile shell growth | | 22 d | | | | |

Table A5. Summary of tropical marine toxicity tests with molluscs, gastropods and cephalopods. Light blue shading identifies SEAM relevant, available test species; dark blue shading signifies SEAM relevant test species which are unlikely to be available; no shading means not relevant to SEAM.

| Species | Lifestage | Endpoint | Acute or Chronic? | Test duration | Temp (°C) | Salinity (‰) | pH | Reference |
|---|---|---|---|---|---|---|---|---|
| **Gastropods** | | | | | | | | |
| *Nerita senegalensis* | 7.5 mm shell length | survival | acute | 24 - 96 h | 22 - 24.5 | 15 | NR | Oyewo and Don-Pedro (2001) |
| *Tympanotonus fuscatus* | 43 mm shell length | survival | acute | 24 - 96 h | 22 - 24.5 | 15 | NR | Oyewo and Don-Pedro (2001) |
| *Strombu gigas (Queen conch)* | embryos | development and survival | sub-chronic | 24 h | 27 | 36.8 | 7.9 | Rumbold and Snedaker (1997) |
| *Nerita chamaeleon (snail)* | NR | growth and survival | chronic | 30 d | 26 ± 0.2 | 32 ± 0.1 | NR | Florence et al. (1994) |
| *Nassarius dorsatus (Dogwhelk snail)* | ≤ 48 h old larvae | growth and survival | chronic | 96 h | 28 | 35 | 8-8.2 | Trenfield et al., (2016) |
| *Babylonia areolata* | adult | survival | acute | 96 h | 25 | ~35 | ~8 | Hajimad and Vedamanikam (2013) |
| | larvae | survival | acute | | | | | Vedamanikamand and Hayimad (2013) |
| *Lithopoma americanum* | adult | behaviour | acute | 24 h | 22 - 23 | 35 | 8.2 | Fong et al. (2015) |
| **Cephalopods** | | | | | | | | |
| *Sepia officinalis* | 1 month old | predatory behaviour and memory | acute | 24 h | 20 | 32 | NR | Di Poi et al. (2013) |

Table A6. Summary of tropical marine toxicity tests with echinoderms. Light blue shading identifies SEAM relevant, available test species; dark blue shading signifies SEAM relevant test species which are unlikely to be available; no shading means not relevant to SEAM.

| Species | Lifestage | Endpoint | Acute or Chronic? | Test duration | Temp (°C) | Salinity (‰) | pH | Reference |
|---|---|---|---|---|---|---|---|---|
| **Sea urchins** | | | | | | | | |
| *Anthocidaris crassipina* | gametes | sperm fertilisation success | chronic | 30 min | 24-26 | 30 | 8 | Vaschenko et al. (1999) |
| | embryos | embryos first cleavage and pluteus larval quality | chronic | 1.5 h - 48 h | | | | |
| | embryos | embryo first cleavage, pluteus | sub-chronic | 27-30 h | 26-28 | NR | NR | Kobayashi and Okamura (2004) |
| | spermatozoa | sperm motility and ultrastructure | sub-chronic | 0, 30 and 60 min | 22 | 30 | NR | Au and Chiang (2000). Au and Reunov (2001) |
| *Diadema savignyi (Long-* | gametes | fertilisation and development | chronic | 48 h | 25 | 34 | 8.1 | Rosen et al. (2015) |
| *Diadema setosum (Long spined sea urchin)* | gametes | sperm fertilisation success | sub-chronic | 1 h | NR | NR | NR | Ramachandran (1997) |
| | embryos | first cleavage | sub-chronic | 1 h | | | | |
| | embryos | gastrulation | sub-chronic | 5 h | | | | |
| | pluteus larvae | development | sub-chronic | 48 h | | | | |
| | fecondated egg | development to pluteus larvae | chronic | 48 h | 27 | 34 | 8.4 | Aquabiotech. Jocelyn Senia, Pers comm. |
| *Echinometra mathaei* (Rock boring sea urchin) | gametes | sperm fertilisation success | sub-chronic | 10 min | 28 | 32.6-35 | 8.2-8.5 | Heslinga (1976) |
| | | embryo early cleavage, skeletal development | chronic | 110 min | | | | |
| | | larval survival and no. swimming | chronic | 96 h | | | | |
| | adult | adult survival | acute | 96 h | | | | |
| | fecondated egg | development to pluteus larvae | chronic | 48 h | 27 | 34 | 8.4 | Aquabiotech. Jocelyn Senia, Pers comm. |
| | gametes | fertilisation | sub-chronic | 1 h | 25 | 35 | 8-8.2 | Geotech. Tristan Stringer, Pers comm. |
| | larvae | larval development | chronic | 72 h | 25 | 35 | 8-8.2 | |
| *Tripneustes gratilla (Collector urchin)* | gametes | fertilization, early mid and late cleavage, blastulation | chronic | 0.5, 3, 6, 9, 12 h | 28 ± 2 | 30 ± 1 | 7 ± 0.5 | Edullantes and Galapate (2014) |
| *Echinodermata lucunter (Rock-boring sea urchin)* | embryos | development to pluteus stage | sub-chronic | 24 h | 26.8 | 36 | 8.1 | Rumbold and Snedaker (1997) |
| *Lytechinus variegatus (Variegated urchin)* | embryos | development to pluteus stage | sub-chronic | 24 h | 27.3 | 35.7 | 8.2 | |
| | larvae (gastrula stage 6 h) | dietary exposure - growth | chronic | 7 - 18 d | 23 - 25 | NR | NR | Brix et al. (2012) |
| *Arabica punctulata* | embryos | development to pluteus stage | chronic | 60 h | NR | NR | NR | Neff et al. (2000) |
| **Star fish** | | | | | | | | |
| *Amphipholis squamata (Brittle star)* | juveniles (disc diameter 0.9 - 1.5 mm) | mortality | acute | 96h | 24-26 | 33-40 | 6.6-8.6 | Black et al. (2015) |
| | juveniles (disc diameter 0.9 - 1.5 mm) | behaviour - 1. ability to right 2. tube feet 3. curling behaviour | sub-chronic | | | | | |
| *Asterias forbesi* | adults | survival | acute | 168 h and 96 h | 20 | 20 | 7.8 ± 0.2 | Eisler and Hennekey (1977) |

Table A7. Summary of tropical marine toxicity tests with annelids. Light blue shading identifies SEAM relevant, available test species; dark blue shading signifies SEAM relevant test species which are unlikely to be available; no shading means not relevant to SEAM.

| Species | Lifestage | Endpoint | Acute or Chronic? | Test duration | Temp (°C) | Salinity (‰) | pH | Reference |
|---|---|---|---|---|---|---|---|---|
| **Polycheates** | | | | | | | | |
| *Hydriodes elegans* | gametes | sperm | chronic | 1 h | 28 ± 1 | 34 ± 0.5 | 8.1 ± 0.1 | Gopalakrishnan et al. (2008) |
| | | egg viability/fertilization | chronic | 1 h | | | | |
| | | embryo development | chronic | 2 h | | | | |
| | "ripe" worms, tube length 5 cm | larval release | chronic | 20 h | | | | |
| | | larval settlement | chronic | 96 h | | | | |
| | | adult survival | acute | 96 h | | | | |
| | trocophore larvae | survival | acute | 48 h | 25 ± 1 | 33 ± 0.5 | 8.1-8.4 | Bao et al. (2011) |
| | eggs | survival | acute | 48 h | NR | 33-34 | NR | Lau et al. (2007) |
| | 2-cells | survival | | | | | | |
| | trocophore larvae | survival | | | | | | |
| | juvenile | survival | | | | | | |
| | adult | survival | | | | | | |
| | egg - juvenile | devlopment | chronic | 17 d | | | | |
| | juvenile | growth and maturation | chronic | 44 d | | | | |
| | post-spawning females | survival and reproduction | chronic | 60 d | | | | |

Table A8. Summary of tropical marine toxicity tests with cnidarians. Light blue shading identifies SEAM relevant, available test species; dark blue shading signifies SEAM relevant test species which are unlikely to be available; no shading means not relevant to SEAM.

| Species | Lifestage | Endpoint | Acute or Chronic? | Test duration | Temp (°C) | Salinity (‰) | pH | Reference |
|---|---|---|---|---|---|---|---|---|
| **Anemones** | | | | | | | | |
| *Aiptasia pulchella* | adult | survival | acute | 96-144 h | 25± 2 | NR | 8.15-8.48 | Howe et al. (2012) |
| | lacerate tentacle | development and survival (asexual reproduction) | chronic | 28 d | 25± 2 | NR | | Howe et al. (2014) A |
| | lacerate tentacle | development and survival | chronic | 14 d | 25± 1 | NR | | Howe et al. (2014) B |
| *Aiptasia sp* | larval and adult | survival | acute | 96 h | 25 ± 1 | 33 ± 0.5 | 8.1-8.4 | Bao et al. (2011) |
| *Aiptasia pallida* [a] | adult | accumulation and enzyme | chronic | 21 d | 20 - 22 | 20 and 25 | 7.8 - 8.1 | Patel and Bielmyer-Fraser (2015) |
| **Corals** | | | | | | | | |
| *Acropora millepora* | gametes | fertilisation | chronic | 4 h | 28 | NR | NR | Negri and Heyward (2001) |
| | larvae | metamorphosis | chronic | 24 h | | | | |
| *Acropora tenuis* | 5-d old larvae | larval settlement | chronic | 48 h | NR | NR | NR | Reichelt-Brushett and Harrison (2000) |
| *Acropora formosa* | coral branches 4-6 cm long | photosynthesis (PAM) | acute | 24 h | 25 ± 1 | 35 | NR | Jones et al. (2003) |
| | coral branches 4-6 cm long | zooxanthellae loss | acute | 48 h | 22-25 | NR | NR | Jones (1997) |
| | adult Coral branches 4-5 cm long | photosynthesis (PAM) | acute | 48 h | NR | NR | NR | Mercurio et al. (2004) |
| | | growth (e.g. bleaching, | acute | 48 h | NR | NR | NR | |
| | | dinoflagellate density ( on | acute | NR | NR | NR | NR | |
| *Acropora tumida* | larvae | survival | acute | 24 h | 25 ± 1 | 33 ± 0.5 | 8.1-8.4 | Bao et al. (2011) |
| *Acropora microphthalma* | gametes | fertilisation | chronic | 4 h | NR | NR | NR | Mercurio et al. (2004) |
| *Lobophytum compactum* [b] | gametes | fertilisation | chronic | 30 min | NR | NR | NR | Reichelt-Brushett and Michalek-Wagner (2005) |
| *Goniastrea aspera* | gametes | fertilisation | chronic | 5 h | NR | NR | NR | Reichelt-Brushett and Harrison (1999) |
| *Montipora aequituberculata* | gametes | fertilisation | chronic | 4 - 6 h | 28 | NR | NR | Negri et al. (2005) |
| | | metamorphosis | chronic | 24 h | 28 | NR | NR | |
| | | larval settlement | chronic | 48 h | 28 | NR | NR | |
| *Montipora digitata* | coral branches 4-6 cm long | photosynthesis (PAM) | acute | 24 h | 25 ± 1 | 35 | NR | Jones et al. (2003) |
| *Montipora capitata* | gametes | fertilisation success | chronic | 3 h | ~22-25 | NR | NR | Hédouin and Gates (2013) |
| *Pocillopora damicornis* | gametes | fertilisation | chronic | 4 - 6 h | 28 | NR | NR | Negri et al. (2005) |
| | | metamorphosis | chronic | 24 h | 28 | NR | NR | |
| | | larval settlement | chronic | 48 h | 28 | NR | NR | |
| | planulae larvae | larval settlement | chronic | 12-96 h | 25-28 | NR | NR | Goh (1991) |
| | | survival | acute | 12-72 h | | | | |
| | coral branches 4-6 cm long | photosynthesis | acute | 96 h | 28 | NR | NR | Negri et al. (2005) |
| *Porites cylindrica* | coral branches 4-6 cm long | photosynthesis (PAM) | acute | 24 h | 25 ± 1 | 35 | NR | Jones et al. (2003) |
| *Seriatopora hystrix* | coral branches 4-6 cm long | photosynthesis (PAM) | acute | 24 h | 25 ± 1 | 35 | NR | Jones et al. (2003) |
| *Xenia elongata* | coral colonies | zooxanthellae loss | acute | 24 - 72 h | 25 | 35 | NR | Studivan et al. (2015) |
| *Heteroxenia fuscescens (Soft coral)* | larvae | planulae metamorphosis and settlement | chronic | 8 d | 25 ± 1 | NR | NR | Kushmaro et al. (1997) |
| *Montastraea faveolata (Mountain coral)* | planulae larvae | survival | sub-chronic | 24 h | 28 | 36 | 7.9 ± 0.2 | Rumbold and Snedaker (1997) |
| | fragments 5 - 10 cm | zooxanthellae loss | chronic | 17 d | 25 - 29 | 33.6 | NR | Jovanovic and Guzman (2014) |

[a] Now known as *Exaiptasia pallida*

[b] Eggs and sperm exposed separately to Cu for 30 min, prior to fertilisation

Table A9. Summary of tropical marine toxicity tests with fish. Light blue shading identifies SEAM relevant, available test species; dark blue shading signifies SEAM relevant test species which are unlikely to be available; no shading means not relevant to SEAM.

| Species | Lifestage | Endpoint | Acute or Chronic? | Test duration | Temp (°C) | Salinity (‰) | pH | Reference |
|---|---|---|---|---|---|---|---|---|
| *Acanthochromis polyacanthus (Damsel fish)* | larvae | imbalance and growth | chronic | 21 d | 28-29 | NR | 7.8-8.1 | Munday et al. (2011) |
| *Chanos chanos* | fingerling | survival | acute | 24 h &96 h | 27 ± 1 | 30 | 8.4 | Cruz and Tamse (1989) |
| | | survival and histopathology | acute | 96 | 26-29 | 30-32 | 7.3-8.4 | Tamse and Gacutan (1994) |
| *Lates calcarifer* | 20-day old juvenile fish | survival | sub-chronic | 96 h – 16 d | 24.5-28 | 20 | NR | Shazili (1995) |
| | 4 month old | survival | sub-chronic | 23d | 24.5-28 | 30 | NR | |
| | juvenile (2 sizes, 11 ± 3, or 24 ± 4) | fry survival, histopathology | acute | 96 h | 28 ± 2 | 26 ± 1 | 8.5± 0.2 | Krishnani et al. (2003) |
| | young males (299 ± 39 g) | induction of enzyme activity (EROD) | chronic | 96 h | 27-28 | 35 | NR | Mercurio et al. (2004) |
| | juvenile | juvenile imbalance, growth | acute | 7 d | 25 ± 1 | NR | NR | ESA SOP 122 |
| *Liza klunzingeri (Mullet)* | NR | survival | acute | 71 1 | 25 ± 2 | NR | 8.2 | Bu-olayan and Thomas (2005) |
| *Liza vaigiensis* | juvenile | survival | acute | 96 h | 20 & 30 | 20 & 36 | NR | Denton and Burdon-jones (1986) |
| *Priopidichthys marianus* | juvenile and adult | survival | acute | 96 h | 20 & 30 | 20 & 36 | NR | |
| *Rivulus marmoratus* | juvenile | survival | acute | 96 h | 26-27 | NR | NR | Lin and Dunson (1993) |
| Mullet (*Mugil* sp.) | fingerling (70 mm) | survival | acute | 24 - 96 h | 22 - 24.5 | 15 | NR | Oyewo and Don-Pedro (2001) |
| *Tilapia guineensis* | fingerling (65 mm) | survival | acute | 24 - 96 h | 22 - 24.5 | 15 | NR | Oyewo and Don-Pedro (2001) |
| Oryzias javanicus (Java medaka) | fertilised egg | hatch success, development | chronic | 15 d | NR | 20 | NR | Ismail and usof (2011) |
| | fertilised egg | survival | chronic | 20 d | 28-30 | 20 | 5.5-6.5 | Yusof et al. (2014) |
| | | hatch success, development | | | | | | |
| | | heart rate | | | | | | |
| *Oryzias melastigma* | <24 h larvae | survival | acute | 96 h | 25 ± 1 | 33 ± 0.5 | 8.1-8.4 | Bao et al. (2011) |
| | adult (2 months old) | growth and bioaccumulation | chronic | 28 d (dietary exposure) | 25 ± 0.5 | 30 | 7.89 ± 0.07 | Wang and Wang (2014) |
| *Amphiprion clarkii (Yellowtail clownfish)* | NR | survival | acute | 96 h | NR | NR | NR | Neff et al. (2000) |
| *Atherinops affinis (Topsmelt fish)* | 9- 15- d old larvae | survival | acute | 96 h | 20 | 34 | NR | Hunt et al. (2002) |
| | early grastula stage embryos | growth, development and survival | chronic | 40 d | | | | |
| *Leiostomus xanthurus (Spot)* | adult | survival | acute | 96 h | 25-26 | 21 | NR | USEPA ecotox database. Ref 3732 Hansen,D.J., 1983. |
| *Menidia peninsulae (Tidewater silverside)* | NR | survival | acute | 96 h | 25 | 20 | NR | |
| *Epinephelus adscensionis (Rock hind fish)* | embryos | hatching succes and survival | sub-chronic | 24 h | 27 | 36.5 | 8.2 ± 0.1 | Rumbold and Snedaker (1997) |
| *Epinephelus cruentatus (Graysby)* | | | | | 27 | 36.2 | 8.0 ± 0.1 | |
| *Cynoscion nebulosus (Spotted sea trout)* | | | | | 28.8 | 36 | 8.1 ± 0.1 | |
| *Ocyurus Chrysurus (Yellow tail snapper)* | | | | | 30 | 36 | 8.1 ± 0.1 | |
| *Terapon jarbua* | NR | NR | NR | 48 h | NR | NR | NR | Krishnakumari et al. (1983) |
| | NR | NR | NR | 72 h | | | | |
| | NR | NR | NR | 96 h | | | | |
| *Kryptolebias marmoratus* | 7-9 d old | survival | acute | 96 h | 24 | 3 - 36 | 7.3 - 8.3 | Bielmyer et al. (2013) |

Table A10. Gap analysis for high quality (QA1) chronic nickel toxicity data for marine waters in the SEAM region

| Taxa | Current SEAM QA1 Ni data | Additional chronic data required[a] | Chronic test available to generate QA1 data? | | Notes on ecological significance in SEAM | Sensitivity range for nickel from other data[b] | Potential test species from acute protocols? | Feasibility of toxicity testing? (i.e. easy, moderate, difficult) | Chronic test method development required, and target species (if known). |
|---|---|---|---|---|---|---|---|---|---|
| | | | Available | Unknown | | | | | |
| Bacteria | 1 | Yes | 0 | 1 | Limited information on diversity or specific ecological importance in SEAM. Live in association with other important key species, mangroves, seagrasses, corals, anemones and fish. | Low | 0 | Easy | Low priority |
| Protozoa | 0 | Yes | 0 | 0 | Important grazers of microbes, most species ubiquitous globally. High diversity of benthic marine flagellates in tropical systems (Larsen and Pattersen, 1990) | Unknown | 0 | Moderate | Low priority, based on sensitivity of other microorganisms to Ni |
| Microalgae | 3 | Maybe | 2 | 3 | Limited information on diversity or specific ecological importance in SEAM. Primary producer, basis of food chain, most species ubiquitous globally. Dinoflagellate algae have important symbiotic relationship with cnidarians (Howe et al., 2012). | Low | 0 | Easy | Medium priority, but should consider growth inhibition tests with isolated coral zooxanthellae |
| Macroalgae | 0 | Yes | 0 | 1 | Low diversity in tropical systems, but important structural function in coral reef building, nitrogen | Unknown | 0 | | Medium priority |

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|---|---|---|---|---|---|---|---|---|---|
| | | | Available | Unknown | | | | | |
| | | | | | fixation and food source for higher trophic organisms as well as epiphytes growing on seagrass and mangroves (Diaz-Pulido and McCook, 2008; Chaves et al., 2013). Red coralline algae (corralinales) and algae of the order Bryopsidophyceae reach peak biodiversity in the tropics (Kerswell, 2006; Hoeksema, 2007). | | | Moderate | |
| Seagrasses | 0 | Yes | 0 | 4 | Philippines, New Guinea and Indonesia are considered to be the centre of global seagrass biodiversity (Spalding et al., 2003). Form a key structural habitat for fish and invertebrates, act as nurseries for fish and crustaceans (Nagelkerken, 2009). | Unknown | 4 (in-situ studies measuring photosynthesis with PAM) | Difficult | Medium priority |
| Mangroves | 0 | Yes | 0 | 0 | Important structural habitat in tropical systems, form important buffering component between rivers and coral reefs, and act as nurseries for fish and | Unknown | 0 | Difficult | Medium priority |

Table A10. Gap analysis for high quality (QA1) chronic nickel toxicity data for marine waters in the SEAM region

| Taxa | Current SEAM QA1 Ni data | Additional chronic data required[a] | Chronic test available to generate QA1 data? | | Notes on ecological significance in SEAM | Sensitivity range for nickel from other data[b] | Potential test species from acute protocols? | Feasibility of toxicity testing? (i.e. easy, moderate, difficult) | Chronic test method development required, and target species (if known). |
|---|---|---|---|---|---|---|---|---|---|
| | | | Available | Unknown | | | | | |
| | | | | | crustaceans (Nagelkerken, 2009). Provide high productivity, abundant detritus and high levels of organic carbon (Cavalcante et al., 2009) Mangrove species diversity is believed to be highest in the Indo-West Pacific (Hoeksema, 2007). | | | | |
| Rotifers | 0 | Yes | 0 | 0 | Very limited information on species diversity and relevance to SEAM, or marine systems in general, predominately freshwater organisms (Fontaneto et al., 2006). Popular food source in aquaculture (Hagiwara et al., 1995). Common strain Brachionus is a tropical marine rotifer that has been collected around Thailand and Fiji (Hagiwara et al., 1995). Rotifer diversity expected to be much higher than what is currently known (JMBA Global Marine Environment). | Unknown | 0 | Difficult | Low priority |
| Crustaceans | 0 | Yes | 4 | 5 | Crustaceans are an important food source for higher trophic organisms and humans. | Broad depending on species. | 5 | Moderate | High priority |

Table A10. Gap analysis for high quality (QA1) chronic nickel toxicity data for marine waters in the SEAM region

| Taxa | Current SEAM QA1 Ni data | Additional chronic data required[a] | Chronic test available to generate QA1 data? | | Notes on ecological significance in SEAM | Sensitivity range for nickel from other data[b] | Potential test species from acute protocols? | Feasibility of toxicity testing? (i.e. easy, moderate, difficult) | Chronic test method development required, and target species (if known). |
|---|---|---|---|---|---|---|---|---|---|
| | | | Available | Unknown | | | | | |
| | | | | | High copepod diversity in tropical Asian region – particularly due to the high association of copepods with corals (Humes, 1994). Copepods are a major food source for tropical fish, form the link between primary producers and higher trophic organisms (Williams et al., 1988). Peracarid crustaceans, mainly amphipods are believed to be the most dominant group of crustaceans in shallow waters including those of the tropics (Thomas, 1993).<br><br>Barnacles, particularly those associated with corals believed to have a high species richness in Indo-Malayan region (Hoeksema, 2007).<br><br>Shrimps and lobsters have also been shown to have high species diversity in Indo-Pacific (Hoeksema, 2007; Roberts et al., 2002). | | | | |

Table A10. Gap analysis for high quality (QA1) chronic nickel toxicity data for marine waters in the SEAM region

| Taxa | Current SEAM QA1 Ni data | Additional chronic data required[a] | Chronic test available to generate QA1 data? | | Notes on ecological significance in SEAM | Sensitivity range for nickel from other data[b] | Potential test species from acute protocols? | Feasibility of toxicity testing? (i.e. easy, moderate, difficult) | Chronic test method development required, and target species (if known). |
|---|---|---|---|---|---|---|---|---|---|
| | | | Available | Unknown | | | | | |
| | | | | | Life cycle of many crustaceans link key habitats – mangroves, seagrasses and coral reefs (Nemeth, 2009). Importance of amphidromous shrimp species, live as adults in freshwater/estuaries, juveniles develop in marine waters before migrating back to freshwater (e.g. Macrobrachium). Common in tropical systems (Kikkert et al., 2009). | | | | |
| Molluscs | 0 | Yes | 5 | 10 | Mollusc diversity is the highest in the tropics, particularly in the Indo-Pacific in coral reef environments, they are also a major human food source in the region. Extremely high number of bivalve molluscs have been found in New Caledonia. (Bouchet et al., 2002). The highest diversity of gastropod molluscs have been found in Nth Australia, New Guinea, Indonesia and the Philippines (Wells, 1990; Roberts, 2002). | High | 4 | Moderate | High priority |

Table A10. Gap analysis for high quality (QA1) chronic nickel toxicity data for marine waters in the SEAM region

| Taxa | Current SEAM QA1 Ni data | Additional chronic data required[a] | Chronic test available to generate QA1 data? | | Notes on ecological significance in SEAM | Sensitivity range for nickel from other data[b] | Potential test species from acute protocols? | Feasibility of toxicity testing? (i.e. easy, moderate, difficult) | Chronic test method development required, and target species (if known). |
|---|---|---|---|---|---|---|---|---|---|
| | | | Available | Unknown | | | | | |
| Cephalopods | 0 | Yes | 0 | 0 | No information on diversity or ecological importance of cephalopods in tropical marine environments, particularly those of SEAM. Mostly large pelagic organisms, highly developed, key predator in marine systems and also prey of larger fish species (Boyle and Rodhouse, 2008). One nautilus species is endemic to New Caledonia and has been shown to accumulate Ni (Bustamante et al., 2000). | Unknown | 1 | Difficult | Low priority |
| Echinoderms | 1 | Yes | 2 | 3 | Important component of coral reefs, high diversity in the tropics, more so the Caribbean than SEAM. Echinoids (sea urchins) dominate the Caribbean. Limited sampling sites in SEAM, but asteroids (starfish) and holothurians (sea cucumber) dominate in Northeast Pacific (Iken et al., 2010). | High | 2 | Moderate | High priority |
| Cnidarians | 1 | Yes | 4-5 | 7 | Anemones distributed throughout sub-tropical and tropical ecosystems (Howe et al., 2014). Not of | Anemones high sensitivity. Corals | 6 | Moderate | High priority |

Table A10. Gap analysis for high quality (QA1) chronic nickel toxicity data for marine waters in the SEAM region

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|---|---|---|---|---|---|---|---|---|---|
| | | | Available | Unknown | | | | | |
| | | | | | commercial importance but hosts for other important species, symbiotic dinoflagellates, bacteria, fish and invertebrates. Species diversity peaks in higher latitudes, rather than the tropics (Fautin et al., 2013).<br><br>Coral diversity is highest in the tropics. Scleractinia corals, Fungiidae and Acropora corals have highest diversity in Indo-Pacific including SE Asia and West Pacific (Hoeksema, 2007). Key structural habitat in tropical systems (Nagelkerken et al., 2000). Importance economically in tourism and recreational activities (Hoeksema, 2007). | Medium – low sensitivity | | | |
| Sponges | 0 | Yes | 0 | 0 | Limited information on sponges in SEAM. Primary consumer, benthic organism. Filter feeders, therefore at high risk of dissolved Ni exposure. Also provide microhabitats for all trophic level taxa from bacteria to fish, important microhabitat for tropical shrimps | Unknown | 0 | Difficult | Medium priority |

Table A10. Gap analysis for high quality (QA1) chronic nickel toxicity data for marine waters in the SEAM region

| Taxa | Current SEAM QA1 Ni data | Additional chronic data required[a] | Chronic test available to generate QA1 data? | | Notes on ecological significance in SEAM | Sensitivity range for nickel from other data[b] | Potential test species from acute protocols? | Feasibility of toxicity testing? (i.e. easy, moderate, difficult) | Chronic test method development required, and target species (if known). |
|---|---|---|---|---|---|---|---|---|---|
| | | | Available | Unknown | | | | | |
| | | | | | (Hoeksema, 2007). High regional diversity of marine sponges in the Carribbean related to adaptability to different habitats and abiotic factors (Wulff, 2005). | | | | |
| Ascidians | 0 | Yes | 0 | 0 | Sedentary filter feeders, accumulate metals, good indicators of pollution and recently exploited for medicinal properties. High diversity in warm tropical waters, particularly on coral reefs of New Caledonia (Monniot et al., 1991). | Unknown | 0 | Difficult | Medium priority |
| Insects | 0 | Yes | 0 | 0 | Heteroptera and Gerromorpha have high species diversity in estuaries and mangroves in the Indo-West Pacific region, predominately live on the surface of the water (Andersen,1999). | Unknown | 0 | Difficult | Low priority |
| Annelids | 0 | Yes | 0 | 0 | Limited information on diversity or ecological relevance in SEAM. Marine worms play critical roles in trophic interactions and affecting biogeochemical cycles | Broad range depending on endpoint | 0 | Moderate | Medium priority |

Table A10. Gap analysis for high quality (QA1) chronic nickel toxicity data for marine waters in the SEAM region

| Taxa | Current SEAM QA1 Ni data | Additional chronic data required[a] | Chronic test available to generate QA1 data? | | Notes on ecological significance in SEAM | Sensitivity range for nickel from other data[b] | Potential test species from acute protocols? | Feasibility of toxicity testing? (i.e. easy, moderate, difficult) | Chronic test method development required, and target species (if known). |
|---|---|---|---|---|---|---|---|---|---|
| | | | Available | Unknown | | | | | |
| | | | | | (Kicklighter and Hay,2006). Believed to be the most important taxon in benthic marine communities and sensitive indicators of environmental pollution (Dean 2008). Predominately benthic organisms. | | | | |
| Fish | 0 | Yes | 1 | 4 | Richest fish diversity found around Eastern Indonesia, New Guinea and the Philippines. Indonesia has the highest concentration of rare and endemic fishes (Randall, 1998). Life cycle of many tropical fish link key habitats – mangroves, seagrasses and coral reefs (Nemeth, 2009). Common in tropical systems are amphidromous fish- adults live in freshwater/estuaries, larvae develop in marine waters before migrating back into freshwater to grow as adults (e.g. goby). Catadromous fish are born in marine waters, then migrate into freshwaters to develop into adults (e.g. eel). Anadromous fish are born in freshwaters, migrate to the ocean as juveniles where they grow into adults | Low | 4 | Difficult | Medium priority |

Table A10. Gap analysis for high quality (QA1) chronic nickel toxicity data for marine waters in the SEAM region

| Taxa | Current SEAM QA1 Ni data | Additional chronic data required[a] | Chronic test available to generate QA1 data? | | Notes on ecological significance in SEAM | Sensitivity range for nickel from other data[b] | Potential test species from acute protocols? | Feasibility of toxicity testing? (i.e. easy, moderate, difficult) | Chronic test method development required, and target species (if known). |
|---|---|---|---|---|---|---|---|---|---|
| | | | Available | Unknown | | | | | |
| | | | | | before returning to freshwater (Fievet et al., 2001) Importance economically as a food source and in tourism and recreational activities (Hoeksema 2007). | | | | |

# Appendix B. Toxicity of Nickel to Marine Microalgae

Table B1. Models and the corresponding Akaike Information Criterion (AIC) values used in the drc package in R. Four different models were fitted to each dataset (species and metal). The model of best fit was chosen based on the lowest AIC value, and by visual assessment of the curve. The selected model (AIC value highlighted in bold) was then used to determine toxicity estimates for each species and metal tested.

| **Species** | ***Symbiodinium*** | | ***C. closterium (F2)*** | | ***C. closterium (G2)*** | | ***T. lutea*** | |
|---|---|---|---|---|---|---|---|---|
| **Metal** | **Nickel** | **Copper** | **Nickel** | **Copper** | **Nickel** | **Copper** | **Nickel** | **Copper** |
| Model | AIC values | | | | | | | |
| Weibull 1.3 | **506** | **266** | **303** | 219 | **613** | 381 | **671** | **363** |
| Weibull 2.3 | 507 | 277 | 316 | **211** | 625 | **337** | 696 | 383 |
| Log Logistic 3 | 506 | 270 | 308 | 214 | 617 | 360 | 673 | 370 |
| Log Logistic 4 | 493 | 266 | 309 | 215 | 548 | 342 | 673 | 383 |

Table B2. Background concentrations of metals in seawater used in microalgae tests. LOD = Limit of detection. Values in bold exceeded the LOD

| **Metal** | **Al** | **As** | **Ba** | **Cd** | **Co** | **Cr** | **Cu** | **Fe** | **Mn** | **Ni** | **Pb** | **Se** | **Zn** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | **(µg/L)** | | | | | | |
| LOD | 0.42 | 2.9 | 0.04 | 0.51 | 0.42 | 0.48 | 0.72 | 0.76 | 0.06 | 0.96 | 2.3 | 4.3 | 0.19 |
| Tests with *C. closterium* and *T. lutea* | <0.42 | **3.0** | **4.0** | <0.51 | <0.42 | **0.53** | <0.72 | **5.7** | **0.18** | <0.96 | 4.1 | <4.3 | **1.7** |
| Tests with *Symbiodinium* | <0.42 | <2.9 | <0.04 | <0.51 | <0.42 | <0.48 | <0.72 | <0.76 | <0.06 | <0.96 | <2.3 | <4.3 | <0.19 |

Table B3. Comparison of nominal and measured dissolved nickel concentrations in 72-h growth rate inhibition toxicity tests with tropical marine microalgae. Numbers rounded to three significant figures.

| Algal species | Test | Nominal nickel (µg/L) | Measured dissolved nickel (µg/L) | | |
|---|---|---|---|---|---|
| | | | Day 0 | Day 3 | Mean |
| **Dinophyaceae** | | | | | |
| *Symbiodinium* sp Freud Clade C | Definitive 1 | 20 | 17 | 20 | 19 |
| | | 30 | 26 | 27 | 27 |
| | | 45 | 43 | 43 | 43 |
| | | 68 | 62 | 65 | 63 |
| | | 101 | 94 | 98 | 96 |
| | | 152 | 144 | 146 | 145 |
| | | 228 | 220 | 224 | 222 |
| | | 342 | 298 | 298 | 298 |
| | | 513 | 488 | 485 | 486 |
| | | 769 | 714 | 714 | 714 |
| | | 1150 | 1070 | 1070 | 1070 |
| | | 1150 | 1600 | 1600 | 1600 |
| | Definitive 2 | 20 | 20 | 20 | 20 |
| | | 30 | 29 | 29 | 28 |
| | | 45 | 41 | 42 | 42 |
| | | 68 | 59 | 62 | 61 |
| | | 101 | 96 | 94 | 94 |
| | | 152 | 148 | 147 | 147 |
| | | 228 | 225 | 226 | 225 |
| | | 342 | 312 | 312 | 307 |
| | | 513 | 439 | 440 | 439 |
| | | 769 | 667 | 672 | 669 |
| | | 1150 | 1030 | 1050 | 1040 |
| | | 1730 | 1530 | 1550 | 1530 |
| | Definitive 3 | 45 | 38 | 38 | 38 |
| | | 68 | 63 | 63 | 63 |
| | | 101 | 94 | 93 | 94 |
| | | 152 | 135 | 137 | 136 |
| | | 228 | 222 | 227 | 225 |
| | | 342 | 296 | 299 | 297 |
| | | 513 | 436 | 446 | 441 |
| | | 769 | 674 | 672 | 673 |
| | | 1150 | 1090 | 1100 | 1100 |
| | | 1730 | 1620 | 1630 | 16204 |
| **Bacillariophyceae** | | | | | |
| *Ceratoneis closterium (F2)* | Range finder 1 | 10 | 10 | 9 | 10 |
| | | 50 | 44 | 42 | 43 |
| | | 100 | 87 | 87 | 87 |
| | | 500 | 489 | 491 | 490 |

Table B3. Comparison of nominal and measured dissolved nickel concentrations in 72-h growth rate inhibition toxicity tests with tropical marine microalgae. Numbers rounded to three significant figures.

| Algal species | Test | Nominal nickel (µg/L) | Measured dissolved nickel (µg/L) | | |
|---|---|---|---|---|---|
| | | | Day 0 | Day 3 | Mean |
| | | 1000 | 968 | 967 | 967 |
| | | 5000 | 4502 | 4542 | 4522 |
| | | 10000 | 8590 | 8650 | 8620 |
| | Definitive 1 | 500 | 458 | 459 | 458 |
| | | 750 | 687 | 685 | 686 |
| | | 1130 | 1030 | 1030 | 1030 |
| | | 1690 | 1600 | 1620 | 1600 |
| | | 2530 | 2440 | 2450 | 2450 |
| | | 3800 | 3610 | 3670 | 3640 |
| | | 5700 | 5460 | 5460 | 5460 |
| | | 8540 | 8180 | 8200 | 8190 |
| | | 12810 | 12460 | 12030 | 12440 |
| | | 19220 | 16210 | 14780 | 16150 |
| | | 28830 | 23110 | 22350 | 24110 |
| | | 43250 | 34110 | 37020 | 35900 |
| *Ceratoneis closterium (G2)* | Definitive 1 | 1000 | 1040 | 1020 | 1030 |
| | | 1500 | 1520 | 1470 | 1500 |
| | | 2250 | 2250 | 2160 | 2200 |
| | | 3380 | 3480 | 3410 | 3450 |
| | | 5060 | 5240 | 5060 | 5150 |
| | | 7590 | 7730 | 7260 | 7490 |
| | | 11390 | 10720 | 10140 | 10430 |
| | | 17090 | 15040 | 14210 | 14620 |
| | | 25630 | 22570 | 21250 | 21910 |
| | | 38440 | 33940 | 32100 | 33020 |
| | | 57670 | 51430 | 48420 | 49930 |
| | Definitive 2 | 1000 | 990 | 986 | 988 |
| | | 1500 | 1460 | 1460 | 1460 |
| | | 2250 | 2110 | 2100 | 2100 |
| | | 3380 | 3270 | 3250 | 32608 |
| | | 4210 | 4080 | 4060 | 40701 |
| | | 5040 | 4910 | 4910 | 4910 |
| | | 6320 | 5920 | 5880 | 5900 |
| | | 7590 | 6920 | 6950 | 6940 |
| | | 11390 | 9620 | 9680 | 9650 |
| | | 17090 | 14420 | 14230 | 14320 |
| | | 25630 | 20060 | 19450 | 19760 |
| | Definitive 3 | 1000 | 993 | 990 | 992 |
| | | 1500 | 1420 | 1440 | 1430 |
| | | 2250 | 2040 | 2120 | 2080 |
| | | 3380 | 3170 | 3170 | 3170 |

Table B3. Comparison of nominal and measured dissolved nickel concentrations in 72-h growth rate inhibition toxicity tests with tropical marine microalgae. Numbers rounded to three significant figures.

| Algal species | Test | Nominal nickel (µg/L) | Measured dissolved nickel (µg/L) | | |
|---|---|---|---|---|---|
| | | | Day 0 | Day 3 | Mean |
| | | 4200 | 4120 | 3820 | 3970 |
| | | 5040 | 4750 | 4640 | 4690 |
| | | 6320 | 5740 | 4660 | 5200 |
| | | 7590 | 6800 | 6480 | 6640 |
| | | 11390 | 9850 | 9190 | 9520 |
| | | 17090 | 14700 | 14180 | 14440 |
| | | 25630 | 21080 | 20610 | 20840 |
| **Coccolithophyceae** | | | | | |
| *Tisochrysis lutea* | Definitive 1 | 50 | 56 | 61 | 60 |
| | | 75 | 77 | 84 | 83 |
| | | 113 | 114 | 121 | 119 |
| | | 169 | 167 | 175 | 175 |
| | | 253 | 253 | 264 | 262 |
| | | 380 | 380 | 389 | 392 |
| | | 570 | 578 | 586 | 590 |
| | | 854 | 835 | 850 | 854 |
| | | 1280 | 1240 | 1300 | 1290 |
| | | 1920 | 1900 | 1880 | 1900 |
| | | 2880 | 2910 | 2890 | 2890 |
| | | 4330 | 4260 | 4240 | 4240 |
| | | 6490 | 6220 | 6400 | 6300 |
| | | 9730 | 9280 | 9330 | 9150 |
| | | 14600 | 13620 | 13300 | 13450 |
| | Definitive 2 | 50 | 44 | 45 | 44 |
| | | 75 | 67 | 67 | 67 |
| | | 113 | 100 | 99 | 100 |
| | | 169 | 173 | 182 | 177 |
| | | 253 | 244 | 250 | 247 |
| | | 380 | 365 | 370 | 368 |
| | | 570 | 545 | 548 | 546 |
| | | 854 | 831 | 859 | 845 |
| | | 1280 | 1230 | 1260 | 1240 |
| | | 1920 | 1800 | 1840 | 1820 |
| | | 2880 | 2940 | 3040 | 2990 |
| | | 4330 | 4340 | 4470 | 4400 |
| | | 6490 | 6450 | 6430 | 6440 |
| | | 9730 | 9480 | 9510 | 9490 |
| | | 14600 | 13930 | 13680 | 13800 |
| | Definitive 3 | 50 | 44 | 45 | 45 |
| | | 75 | 67 | 70 | 69 |
| | | 113 | 102 | 101 | 101 |

Table B3. Comparison of nominal and measured dissolved nickel concentrations in 72-h growth rate inhibition toxicity tests with tropical marine microalgae. Numbers rounded to three significant figures.

| Algal species | Test | Nominal nickel (µg/L) | Measured dissolved nickel (µg/L) | | |
|---|---|---|---|---|---|
| | | | Day 0 | Day 3 | Mean |
| | | 169 | 165 | 171 | 168 |
| | | 253 | 253 | 251 | 252 |
| | | 380 | 372 | 368 | 370 |
| | | 570 | 558 | 558 | 558 |
| | | 854 | 832 | 833 | 833 |
| | | 1280 | 1050 | 1050 | 10509 |
| | | 1920 | 1980 | 2000 | 1990 |
| | | 2880 | 2700 | 2720 | 2710 |
| | | 4330 | 4250 | 4280 | 4260 |
| | | 6490 | 6520 | 6450 | 6490 |
| | | 9730 | 9200 | 9200 | 9200 |
| | | 14600 | 14200 | 13820 | 14010 |

Table B4. Comparison of nominal and measured dissolved copper concentrations in 72-h growth rate inhibition toxicity tests with tropical marine microalgae. Numbers rounded to three significant figures.

| Algal species | Test | Nominal copper (µg/L) | Measured dissolved copper (µg/L) | | |
|---|---|---|---|---|---|
| | | | Day 0 | Day 3 | Mean |
| **Dinophyaceae** | | | | | |
| *Symbiodinium* sp. Freud Clade C | Definitive 1 | 2 | 0.87 | 0.78 | 0.83 |
| | | 4 | 2.6 | 2.0 | 2.3 |
| | | 6 | 3.8 | 3.6 | 3.7 |
| | | 8 | 5.2 | 5.1 | 5.2 |
| | Definitive 2 | 2 | 0.6 | 1.9 | 1.4 |
| | | 4 | 1.8 | 1.4 | 1.5 |
| | | 6 | 3.0 | 2.8 | 2.8 |
| | | 8 | 4.5 | 4.2 | 4.3 |
| | | 10 | 6.2 | 5.2 | 5.6 |
| | Definitive 3 | 4 | 2.1 | 1.1 | 1.6 |
| | | 8 | 4.8 | 4.6 | 4.7 |
| | | 10 | 5.6 | 4.7 | 5.2 |
| | | 12 | 8.1 | 7.2 | 7.7 |
| **Bacillariophyceae** | | | | | |
| *Ceratoneis closterium (F2)* | Range finder 1 | 1 | 0.53 | 0.41 | 0.47 |
| | | 5 | 4.2 | 3.4 | 3.8 |
| | | 10 | 8.8 | 7.1 | 7.9 |
| | | 20 | 17 | 14 | 15 |
| | | 40 | 33 | 28 | 30 |
| | Definitive 1 | 1 | 1.0 | 0.3 | 0.6 |
| | | 3 | 2.7 | 1.5 | 2.1 |
| | | 6 | 6.0 | 2.9 | 4.4 |
| | | 12 | 9.8 | 6.9 | 8.4 |
| | | 18 | 16 | 12 | 14 |
| *Ceratoneis closterium (G2)* | Definitive 1 | 0.25 | 0.75 | 0.55 | 0.65 |
| | | 0.5 | 0.6 | 0.5 | 0.5 |
| | | 1 | 1.0 | 0.7 | 0.8 |
| | | 2 | 1.9 | 1.2 | 1.6 |
| | | 4 | 3.3 | 2.4 | 2.8 |
| | | 8 | 6.5 | 5.2 | 5.9 |
| | | 12 | 9.3 | 7.5 | 8.4 |
| | Definitive 2 | 0.5 | 0.69 | 0.34 | 0.52 |
| | | 1 | 0.9 | 0.5 | 0.7 |
| | | 2 | 1.7 | 1.2 | 1.5 |
| | | 4 | 3.2 | 2.5 | 2.8 |
| | | 8 | 6.3 | 5.4 | 5.8 |
| | | 12 | 11 | 8.1 | 9.6 |
| | | 20 | 16 | 13 | 15 |
| | Definitive 3 | 0.50 | 0.58 | 0.25 | 0.41 |

Table B4. Comparison of nominal and measured dissolved copper concentrations in 72-h growth rate inhibition toxicity tests with tropical marine microalgae. Numbers rounded to three significant figures.

| Algal species | Test | Nominal copper (µg/L) | Measured dissolved copper (µg/L) | | |
|---|---|---|---|---|---|
| | | | Day 0 | Day 3 | Mean |
| | | 1 | 0.9 | 0.5 | 0.7 |
| | | 2 | 1.7 | 1.1 | 1.4 |
| | | 4 | 3.6 | 2.2 | 2.9 |
| | | 8 | 6.4 | 5.6 | 6.0 |
| | | 12 | 12 | 7.8 | 9.9 |
| | | 20 | 16 | 13 | 15 |
| **Coccolithophyceae** | | | | | |
| *Tisochrysis lutea* | Definitive 1 | 0.50 | 0.51 | -0.06 | 0.22 |
| | | 1 | 1.1 | 0.1 | 0.6 |
| | | 2 | 2.0 | 1.0 | 1.5 |
| | | 4 | 3.8 | 2.8 | 3.3 |
| | | 8 | 7.5 | 6.0 | 6.7 |
| | | 12 | 15 | 12 | 13 |
| | | 16 | 17 | 14 | 15 |
| | Definitive 2 | 0.50 | 0.80 | 0.80 | 0.80 |
| | | 1 | 1.3 | 1.3 | 1.3 |
| | | 2 | 2.7 | 2.4 | 2.5 |
| | | 4 | 4.5 | 6.9 | 5.7 |
| | | 8 | 8.3 | 7.6 | 7.9 |
| | | 12 | 12 | 10 | 11 |
| | | 16 | 16 | 14 | 15 |
| | Definitive 3 | 0.50 | 0.83 | 0.67 | 0.75 |
| | | 1 | 1.3 | 1.1 | 1.2 |
| | | 2 | 2.3 | 2.0 | 2.2 |
| | | 4 | 4.2 | 3.8 | 4.0 |
| | | 8 | 7.9 | 6.9 | 7.4 |
| | | 12 | 12 | 11 | 11 |
| | | 16 | 16 | 14 | 15 |

# Appendix C. Assessing the Chronic Toxicity of Nickel to a Tropical Marine Gastropod and two Crustaceans

Table C1. Models and the corresponding Akaike Information Criterion (AIC) values used in the drc package in R. Three different models were fitted to each data set (species and metal). The model of best fit was chosen based on the lowest AIC value, and by visual assessment of the curve. The selected model was then used to determine toxicity estimates for each species and metal tested.

| **Species** | ***Nassarius dorsatus*** | | ***Amphibalanus amphitrite*** | | ***Acartia sinjiensis*** | |
|---|---|---|---|---|---|---|
| **Metal** | **Nickel** | **Copper** | **Nickel** | **Copper** | **Nickel** | **Copper** |
| Model | AIC values | | | | | |
| Weibull 1.3 | 678 | 128 | 738 | 145 | 632 | 653 |
| Log Logistic 3 | 682 | 129 | 742 | 148 | 633 | 649 |
| Log Logistic 4 | 678 | 192 | 741 | 146 | 832 | 845 |

Table C2. Background concentrations of metals in seawater used in snail tests. LOD = Limit of detection. Values in bold exceeded the LOD.

| **Metal** | **Al** | **Ba** | **Cd** | **Co** | **Cr** | **Cu** | **Mn** | **Ni** | **Pb** | **Se** | **V** | **Zn** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | **(µg/L)** | | | | | | |
| LOD | 0.30 | 0.07 | 0.20 | 1.0 | 0.77 | 0.50 | 0.06 | 2.0 | 2.1 | 2.6 | 0.20 | 0.18 |
| Ni test 1 | <0.30 | **2.0** | <0.20 | <1.0 | <0.77 | <0.50 | **0.70** | <2.0 | <2.1 | **4.3** | <0.20 | **5.5** |
| Ni test 2 | <0.30 | **1.8** | <0.20 | <1.0 | <0.77 | <0.50 | **1.0** | <2.0 | <2.1 | <2.5 | <0.20 | <0.18 |
| Ni test 3 | <0.30 | **1.8** | <0.20 | <1.0 | <0.77 | <0.50 | **0.93** | <2.0 | <2.1 | <2.5 | <0.20 | **1.1** |
| Ni test 4 | <0.30 | **1.9** | <0.20 | <1.0 | <0.77 | <0.50 | **0.82** | <2.0 | <2.1 | <2.5 | <0.20 | **5.2** |
| Cu test 1 | <0.30 | **1.9** | <0.20 | <1.0 | <0.77 | <0.50 | **1.1** | <2.0 | <2.1 | **3.5** | <0.20 | <0.18 |

Table C3. Background concentrations of metals in sea water used in barnacle tests. LOD = Limit of detection. Values in bold exceeded the LOD.

| **Metal** | **Al** | **As** | **Ba** | **Cd** | **Co** | **Cr** | **Cu** | **Fe** | **Mn** | **Ni** | **Pb** | **Se** | **V** | **Zn** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | **(µg/L)** | | | | | | |
| LOD | 0.23 | 2.0 | 2.1 | 0.14 | 0.29 | 0.46 | 0.45 | 0.78 | 0.18 | 1.2 | 2.7 | 2.5 | 1.1 | 0.68 |
| Ni test 1 | <0.23 | **3.8** | **2.2** | <0.14 | <0.29 | <0.46 | <0.45 | <0.78 | <0.18 | <1.2 | <2.7 | <2.5 | **2.7** | <0.68 |
| Ni test 2 | <0.23 | **4.7** | **2.8** | <0.14 | <0.29 | <0.46 | <0.45 | <0.78 | <0.18 | <1.2 | <2.7 | <2.5 | <1.1 | <0.68 |
| Ni test 3 | <0.23 | **4.8** | <2.1 | <0.14 | <0.29 | <0.46 | <0.45 | <0.78 | <0.18 | <1.2 | <2.7 | **2.6** | <1.1 | <0.68 |
| Ni test 4 | **4.5** | <2.0 | **3.8** | <0.14 | <0.29 | <0.46 | <0.45 | <0.78 | <0.18 | <1.2 | <2.7 | <2.5 | <1.1 | <0.68 |
| Cu test 1 | <0.23 | **4.0** | <2.1 | <0.14 | <0.29 | <0.46 | <0.45 | <0.78 | <0.18 | <1.2 | <2.7 | <2.5 | <1.1 | <0.68 |

Table C4. Background concentrations of metals in sea water used in copepod tests. LOD = Limit of detection. Values in bold exceeded the LOD. Each test included Ni and Cu treatments.

| **Metal** | **Ag** | **Al** | **As** | **Ba** | **Cd** | **Co** | **Cr** | **Cu** | **Fe** | **Mn** | **Ni** | **Pb** | **Se** | **V** | **Zn** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | **(µg/L)** | | | | | | | |
| LOD | 2.0 | 0.36 | 3.5 | 0.13 | 0.13 | 0.74 | 0.79 | 1.1 | 0.35 | 0.12 | 0.92 | 3.2 | 4.6 | 0.59 | 0.15 |
| Test 1 | <2.0 | <0.36 | <3.5 | **0.75** | <0.13 | <0.74 | <0.79 | <1.1 | <0.35 | <1.2 | <0.92 | <3.2 | <4.6 | <0.59 | **6.6** |
| Test 2 | <2.0 | <0.36 | <3.5 | <0.13 | <0.13 | <0.74 | <0.79 | <1.1 | <0.35 | **0.22** | <0.92 | <3.2 | <4.6 | <0.59 | <0.15 |
| Test 3 | <2.0 | <0.36 | <3.5 | <0.13 | <0.13 | <0.74 | <0.79 | <1.1 | <0.35 | <1.2 | <0.92 | <3.2 | <4.6 | <0.59 | <0.15 |

Table C5. Comparison of nominal and measured dissolved nickel and copper concentrations in 4-day toxicity tests with the snail *Nassarius dorsatus*. TWA = Time weighted average. Numbers rounded to three significant figures.

| Test | Nominal (µg/L) | Measured dissolved (µg/L) | | | |
|---|---|---|---|---|---|
| | | Day 0 | Day 2 | Day 4 | TWA |
| Ni test 1 | 200 | 193 | 193 | 194 | 194 |
| | 240 | 234 | 233 | 238 | 235 |
| | 280 | 274 | 273 | 280 | 275 |
| | 320 | 313 | 316 | 318 | 324 |
| | 360 | 349 | 352 | 351 | 342 |
| | 400 | 388 | 392 | 391 | 390 |
| Ni test 2 | 150 | 145 | 148 | 146 | 147 |
| | 250 | 247 | 252 | 248 | 250 |
| | 350 | 344 | 347 | 343 | 345 |
| | 450 | 442 | 441 | 444 | 442 |
| | 600 | 587 | 591 | 593 | 590 |
| | 900 | 882 | 882 | 886 | 883 |
| Ni test 3 | 50 | 48 | 49 | 47 | 48 |
| | 100 | 100 | 97 | 96 | 97 |
| | 500 | 493 | 489 | 482 | 488 |
| | 700 | 689 | 685 | 685 | 686 |
| | 1000 | 982 | 974 | 975 | 976 |
| | 1500 | 1480 | 1450 | 1480 | 1460 |
| Ni test 4 | 80 | 75 | 78 | 75 | 76 |
| | 200 | 198 | 195 | 195 | 196 |
| | 400 | 392 | 386 | 386 | 387 |
| | 800 | 786 | 768 | 770 | 773 |
| | 1200 | 1170 | 1150 | 1160 | 1160 |
| | 1400 | 1370 | 1340 | 1340 | 1350 |
| Cu test 1 | 2 | 2.1 | <0.5 | 0.7 | 1.4 |
| | 4 | 3.6 | 0.2 | 2.1 | 2.9 |
| | 8 | 7.3 | 3.5 | 4.8 | 6.0 |
| | 12 | 11 | 7.5 | 7.9 | 9.2 |
| | 20 | 17 | 13 | 11 | 14 |

Table C6. Comparison of nominal and measured nickel and copper concentrations in 4-day toxicity tests with the barnacle *Amphibalanus amphitrite*

| | Nominal | Measured dissolved (µg/L) | | |
|---|---|---|---|---|
| **Test** | **(µg/L)** | **Day 0** | **Day 4** | **Mean** |
| Ni test 1 | 50 | 44 | 44 | 44 |
| | 100 | 92 | 89 | 90 |
| | 150 | 137 | 137 | 137 |
| | 200 | 189 | 183 | 186 |
| | 500 | 461 | 466 | 463 |
| Ni test 2 | 50 | 49 | 45 | 47 |
| | 75 | 74 | 68 | 71 |
| | 150 | 144 | 136 | 140 |
| | 300 | 305 | 290 | 298 |
| | 400 | 402 | 383 | 392 |
| Ni test 3 | 60 | 55 | 53 | 54 |
| | 120 | 112 | 109 | 110 |
| | 200 | 189 | 185 | 187 |
| | 280 | 283 | 276 | 280 |
| | 380 | 379 | 372 | 375 |
| Ni test 4 | 90 | 84 | 76 | 80 |
| | 180 | 173 | 161 | 167 |
| | 250 | 241 | 228 | 235 |
| | 350 | 368 | 344 | 356 |
| Cu test 1 | 20 | 15 | 7 | 11 |
| | 40 | 33 | 18 | 26 |
| | 60 | 50 | 28 | 39 |
| | 80 | 65 | 40 | 53 |
| | 100 | 87 | 55 | 71 |

Table C7. Comparison of nominal and measured dissolved nickel and copper concentrations in 3-day toxicity tests with the copepod *Acartia sinjiensis*. TWA = Time weighted average. Day 2B = samples taken on Day 2 before renewal of test solutions. Day 2A = samples taken on Day 2 after renewal of test solutions. NA = not analysed.

| Test | Nominal (µg/L) | Measured dissolved (µg/L) | | | | |
|---|---|---|---|---|---|---|
| | | Day 0 | Day 2B | Day 2A | Day 3 | TWA |
| Nickel | | | | | | |
| Test 1 | 4 | 3.5 | 4.2 | 3.3 | 2.9 | 4.0 |
| | 6 | 6.3 | 5.6 | 5.4 | 5.9 | 6.0 |
| | 8 | 7.2 | 7.2 | 7.4 | 8.5 | 7.9 |
| | 12 | 13 | 12 | 12 | 11 | 12 |
| | 16 | 16 | 16 | 15 | 15 | 16 |
| Test 2 | 4 | 3.6 | 3.5 | 4.1 | 3.4 | 3.6 |
| | 6 | 4.9 | 5.6 | 5.2 | 4.7 | 5.5 |
| | 8 | 6.5 | 6.3 | 6.6 | 6.0 | 6.3 |
| | 10 | 8.7 | 7.8 | 8.7 | 8.5 | 8.4 |
| | 12 | 10 | 10 | 9 | 11 | 11 |
| Test 3 | 5 | 5.0 | 4.5 | 4.3 | 5.0 | 5.0 |
| | 7 | 6.1 | 5.4 | 6.9 | 5.7 | 6.0 |
| | 9 | 7.0 | 6.4 | 7.2 | 7.8 | 7.2 |
| | 11 | 8.3 | 8.5 | 9.6 | 8.9 | 8.9 |
| | 13 | 11 | 10 | 11 | 11 | 11 |
| Copper | | | | | | |
| Test 1 | 1 | 1.2 | 0.7 | 1.1 | 0.8 | 0.9 |
| | 3 | 2.8 | 1.3 | 2.2 | 2.2 | 2.1 |
| | 6 | 3.4 | 1.8 | 2.9 | 2.8 | 2.7 |
| | 9 | 5.9 | 2.8 | 5.1 | 4.1 | 4.5 |
| | 12 | 8.4 | 4.7 | 7.7 | 6.3 | 6.8 |
| Test 2 | 1 | 0.9 | NA | 1.0 | 0.8 | 0.9 |
| | 3 | 3.0 | 1.3 | 2.2 | 2.0 | 2.1 |
| | 6 | 2.9 | 1.1 | 2.6 | 2.3 | 2.2 |
| | 9 | 5.0 | 2.9 | 4.3 | 3.6 | 3.9 |
| | 12 | 6.8 | 3.7 | 6.8 | 5.5 | 5.6 |
| Test 3 | 1 | 0.8 | 0.4 | 0.9 | 0.5 | 0.6 |
| | 2 | 1.5 | 0.6 | 1.5 | 1.1 | 1.1 |
| | 4 | 3.3 | 1.6 | 2.7 | 2.2 | 2.4 |
| | 6 | 3.8 | 2.2 | 3.7 | 2.6 | 3.1 |
| | 8 | 5.8 | 3.1 | 4.8 | 3.4 | 4.3 |

# Appendix D. Inhibition of Coral Fertilisation following Exposure to Nickel and Copper

Table D1. Environmental concentrations of nickel around tropical coral reef locations in Asia Pacific.

| Location | Sample | Nickel | Study |
|---|---|---|---|
| Compiled from Thailand, Australia, Philippines | Sediment | 74-123 mg/kg | Peters et al., 1997 |
| Sabah, Malaysia | Seawater | 0.4-1.9 µg/L | Mokhtar et al., 2012 |
| | Sediment | 20-60 mg/kg | |
| Manila Bay, Philippines | Sediment | 10-19 mg/kg | Prudente et al., 1994 |
| Bay of Bengal, India | Seawater | 0.021-3.56 µg/L | Srichandan et al., 2016 |
| New Caledonia | Sediment | 5-900 mg/kg | Hedouin et al., 2009 |
| Noumea, New Caledonia | Sediment | 28-1879 mg/kg | Monniot et al., 1991 |
| Boulari Bay and Saint Marie Bay, Noumea, New Caledonia | Sediment | 7000 mg/kg | Fernandez et al., 2006 |
| Townsville, Australia | Sediment | 5.5-285 nM/g | Esslemont, 2000 |

dw = dry weight

Table D2. A list of the models and the corresponding Akaike Information Criterion (AIC) values used in the drc package in R. Four different models were fitted to each data set (species and metal). The model of best fit was chosen based on the lowest AIC value, and by visual assessment of the curve. The selected model was then used to determine toxicity estimates for each species and metal tested.

| **Species** | ***Acropora aspera*** | | ***Acropora digitifera*** | | ***Platygyra daedalea*** | |
|---|---|---|---|---|---|---|
| **Metal** | **Nickel** | **Copper** | **Nickel** | **Copper** | **Nickel** | **Copper** |
| Model | AIC values | | | | | |
| Weibull 1.3 | 210 | 185 | 232 | Not tested | 111 | 156 |
| Weibull 2.3 | 211 | 183 | 223 | | 268 | 157 |
| Log Logistic 3 | 211 | 185 | 227 | | 112 | 157 |
| Log Logistic 4 | 212 | 185 | 223 | | 112 | 159 |

NT = not tested

Table D3. Background concentrations of metals in seawater used in toxicity tests

| **Metal** | **Fe** | **Mo** | **Mn** | **Al** | **As** | **Ba** | **Cd** | **Co** | **Cr** | **Cu** | **Ni** | **Se** | **V** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | **(µg/L)** | | | | | | |
| *LOD* | *0.47* | *0.68* | *0.60* | *0.51* | *4.4* | *0.20* | *0.35* | *0.55* | *0.61* | *0.80* | *2.1* | *3.5* | *0.69* |
| **Test no.** | | | | | | | | | | | | | |
| 1 | <0.47 | <0.68 | <0.60 | <0.51 | <4.4 | <0.2 | <0.35 | <0.55 | <0.61 | <0.80 | <2.1 | <3.5 | <0.69 |
| 2 | <0.47 | **0.86** | <0.60 | <0.51 | <4.4 | <0.2 | <0.35 | <0.55 | <0.61 | **1.2** | <2.1 | <3.5 | <0.69 |

Figures in bold indicate values above the limit of detection

Table D4. Nominal and measured metal concentrations used in 5-h fertilisation toxicity tests with scleractinian corals. Note, the same concentration range was tested for *A. aspera* and *A. digitifera* so only one set of metal sub-samples was analysed. Numbers rounded to three significant figures

| **Species** | **Nominal** | **Dissolved metal measured t=0 h** | **Dissolved metal measured t=5 h** | **Metal loss over 5 h** |
|---|---|---|---|---|
| | | **(µg/L)** | | **%** |
| | **Nickel** | | | |
| *Acropora aspera* and *Acropora digitifera* | 300 | 285 | 271 | 4.9 |
| | 1000 | 950 | 938 | 1.2 |
| | 2500 | 2430 | 2420 | 0.3 |
| | 5000 | 4790 | 4680 | 2.2 |
| | 10000 | 9360 | 9090 | 2.8 |
| *Platygyra daedalea* | 100 | 93 | 91 | 2.2 |
| | 500 | 445 | 433 | 2.6 |
| | 1000 | 932 | 907 | 2.7 |
| | 2500 | 2350 | 2370 | -0.9 |
| | 5000 | 4630 | 4600 | 0.8 |
| | **Copper** | | | |
| *Acropora aspera* and *Acropora digitifera* | 10 | 7.8 | 4.0 | 48 |
| | 20 | 17 | 7.8 | 55 |
| | 40 | 34 | 19 | 45 |
| | 80 | 71 | 37 | 48 |
| *Platygyra daedalea* | 10 | 6.4 | 3.7 | 42 |
| | 20 | 11 | 7.9 | 26 |
| | 40 | 15 | 22 | -49 |
| | 80 | 39 | 35 | 9.0 |

### Larval survival from fertilisation

Table D5. Toxicity test conditions for larval survival experiment with *Platygyra daedalea*

| **Toxicity test parameters** | |
|---|---|
| Temperature | 25 ± 2°C |
| pH | 8.1 ± 0.1 |
| Salinity | 34 ± 1‰ |
| Conductivity | 51 ± 1 mS/cm |
| Dissolved oxygen | >8 mg/L |
| Light | Ambient natural light |
| Test type | Static, renewal at 10 h and 36 h (after fertilisation) |
| Test duration | 72 h |
| Test chamber | 20 mL glass scintillation vials |
| Test solution volume | 20 mL |
| Age of test organism | Gametes |
| Initial spermatozoa density | 2 x$10^6$/mL |
| Initial no. eggs | ~100 |
| No. replicate chambers per treatment | 5 |
| Control/diluent water | Natural, sperm-free seawater |
| Test endpoint | Larval survival |

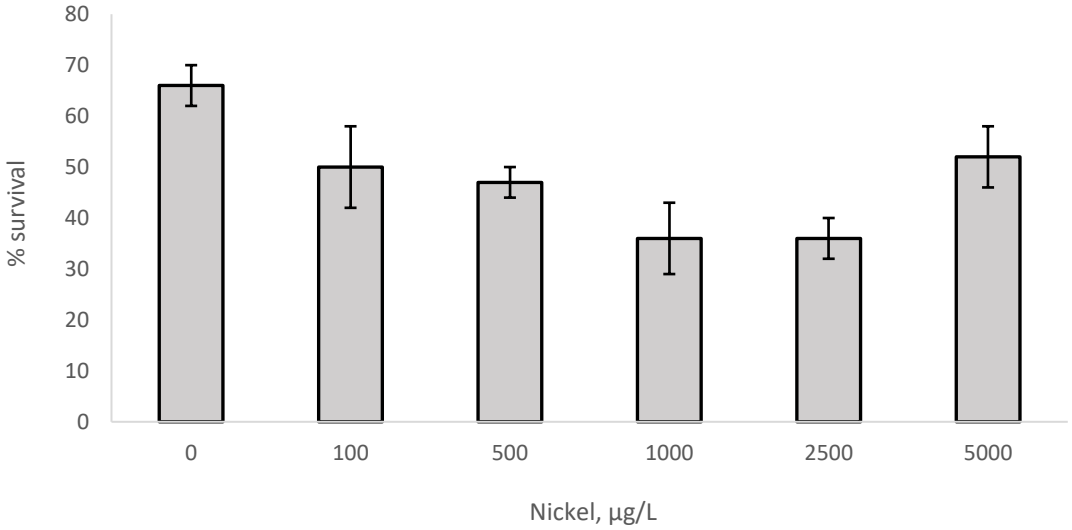



Figure D1. The effect of Ni on larval survival of *Platygyra daedalea* following 72-h exposure from fertilisation

# Appendix E. Toxicity of Nickel and Copper to the Adult Coral *Acropora muricata* and its Microbiome

Table E1. PCR conditions for amplification of DNA using various primers

| Primer set | PCR conditions | | |
|---|---|---|---|
| | Denature (°C, min) | Annealing (°C, s) | Extension (°C, min) |
| 18S | 95, 2 | 40 cycles:<br>95, 30<br>58, 30<br>72, 60 | 72, 7 |
| 16S | 94, 3 | 35 cycles:<br>94, 45<br>50, 60<br>72, 90 | 72, 10 |

Table E2. Total number of reads and operational taxonomic units (OTUs) prior to and after filtering the data in preparation for statistical analysis

| | Before filtering | | After filtering | |
|---|---|---|---|---|
| Dataset | OTUs | Reads | OTUs | Reads |
| 18S rDNA | 681 | 3290956 | 30 | 2387661 |
| 16S rDNA | 2163 | 1859154 | 209 | 1443382 |

Table E3. Background concentrations of metals in filtered seawater used to make treatment solutions each day during the 4-day exposures. LOD = Limit of detection. Values in bold exceeded the LOD.

| | **Al** | **As** | **Ba** | **Be** | **Cd** | **Co** | **Cr** | **Cu** | **Fe** | **Fe** | **Mn** | **Ni** | **Pb** | **Se** | **Zn** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | **(µg/L)** | | | | | | | |
| LOD | 0.52 | 0.76 | 0.05 | 0.003 | 0.56 | 0.24 | 1.1 | 0.31 | 0.60 | 3.9 | 0.21 | 1.8 | 1.9 | 3.0 | 0.12 |
| t=0 | **1.3** | <0.76 | **0.51** | <0.003 | <0.56 | <0.24 | <1.1 | **1.3** | **3.6** | <3.9 | <0.21 | <1.8 | <1.9 | <3.0 | <0.12 |
| t=1 d | **1.0** | <0.76 | **0.61** | <0.003 | <0.56 | <0.24 | <1.1 | <0.31 | **4.3** | **4.1** | <0.21 | <1.8 | <1.9 | <3.0 | <0.12 |
| t=2 d | **2.6** | <0.76 | **0.54** | <0.003 | <0.56 | <0.24 | <1.1 | **2.1** | <0.60 | <3.9 | <0.21 | <1.8 | <1.9 | <3.0 | **2.0** |
| t=3 d | **0.5** | <0.76 | **0.17** | <0.003 | <0.56 | <0.24 | <1.1 | <0.31 | <0.60 | <3.9 | <0.21 | <1.8 | **2.1** | <3.0 | <0.12 |

Table E4. Concentrations of dissolved and total nickel and copper, measured in the treatment tanks on Days 0-3. Sub-samples were taken immediately after treatment solutions were made. NM = not measured. These treatments were sacrificed at 36 h and so treatment solutions were not made on Days 2-3.

| | Day | | | |
|---|---|---|---|---|
| **Treatment** | **0** | **1** | **2** | **3** |
| **Dissolved metal (<0.45 µm) (µg/L )** | | | | |
| Control | 0.0 | 0.0 | 0.8 | 0.3 |
| Ni 50 | 47 | 47 | 45 | 45 |
| Ni 100 | 92 | 92 | 93 | 93 |
| Ni 500 | 493 | 474 | 467 | 457 |
| Ni 1000 | 914 | 910 | 912 | 902 |
| Ni 10000 | 9170 | 9070 | NM | NM |
| Control | 1.3 | 1.0 | 2.1 | 0.4 |
| Cu 5 | 5.6 | 4.6 | 4.7 | 4.6 |
| Cu 20 | 17 | 17 | NM | NM |
| Cu 50 | 46 | 43 | NM | NM |
| Cu 100 | 89 | 85 | NM | NM |
| **Total metal (µg/L)** | | | | |
| Control | 0.0 | 0.2 | 0.6 | 0.6 |
| Ni 50 | 48 | 47 | 49 | 46 |
| Ni 100 | 96 | 92 | 94 | 92 |
| Ni 500 | 513 | 485 | 479 | 468 |
| Ni 1000 | 939 | 924 | 921 | 914 |
| Ni 10000 | 9600 | 9390 | NM | NM |
| Control | 1.2 | 0.9 | 1.4 | 0.6 |
| Cu 5 | 4.6 | 5.8 | 4.7 | 4.8 |
| Cu 20 | 19 | 27 | NM | NM |
| Cu 50 | 48 | 46 | NM | NM |
| Cu 100 | 88 | 89 | NM | NM |

Table E5. Concentrations of total nickel and copper, measured in the test chambers on Day 0 (initial) and at 36 or 96 h.

| | Initial | | 36 or 96 h | |
|---|---|---|---|---|
| **Treatment** | **Mean[a]** | **SD[a]** | **Mean[a]** | **SD[a]** |
| | | **Total (µg/L)** | | |
| Control | 0.0 | 0.7 | 0.4 | 0.7 |
| Ni 50 | 43 | 0.6 | 46 | 0.3 |
| Ni 100 | 87 | 0.4 | 91 | 0.8 |
| Ni 500 | 483 | 6.9 | 464 | 5.1 |
| Ni 1000 | 894 | 11 | 912 | 3.3 |
| Ni 10000 [b] | 8960 | 85 | 9220 | 75 |
| Control | 3.2 | 0.5 | 0.8 | 0.0 |
| Cu 5 | 4.1 | 0.3 | 3.2 | 0.2 |
| Cu 20 [b] | 13 | 0.5 | 12 | 0.9 |
| Cu 50 [b] | 35 | 0.9 | 40 | 1.5 |
| Cu 100 [b] | 69 | 2.0 | 70 | 1.6 |

[a] Taken from 4 replicate chambers
[b] These treatments were sacrificed and samples taken for analysis at 36 h (not at 96 h), due to bleaching.

Table E6. Coral watch health chart scores for coral fragments following exposure to nickel and copper for 36-96 h. (https://www.coralwatch.org/web/guest/coral-health-chart)

| **Treatment** | **Score** | **Description** |
|---|---|---|
| Control | D5-D6 | Healthy branching coral |
| 4 µg Cu/L | D5-D6 | Healthy branching coral |
| 11 µg Cu/L | D3 | Slightly bleached |
| 32 µg Cu/L | D1 | Severely bleached |
| 65 µg Cu/L | D2 | Moderately bleached |
| 45 µg Ni/L | D5-D6 | Healthy branching coral |
| 90 µg Ni/L | D1 | Severely bleached |
| 470 µg Ni/L | D2 | Moderately bleached |
| 900 µg Ni/L | D2 | Moderately bleached |
| 9050 µg Ni/L | D2 | Moderately bleached |

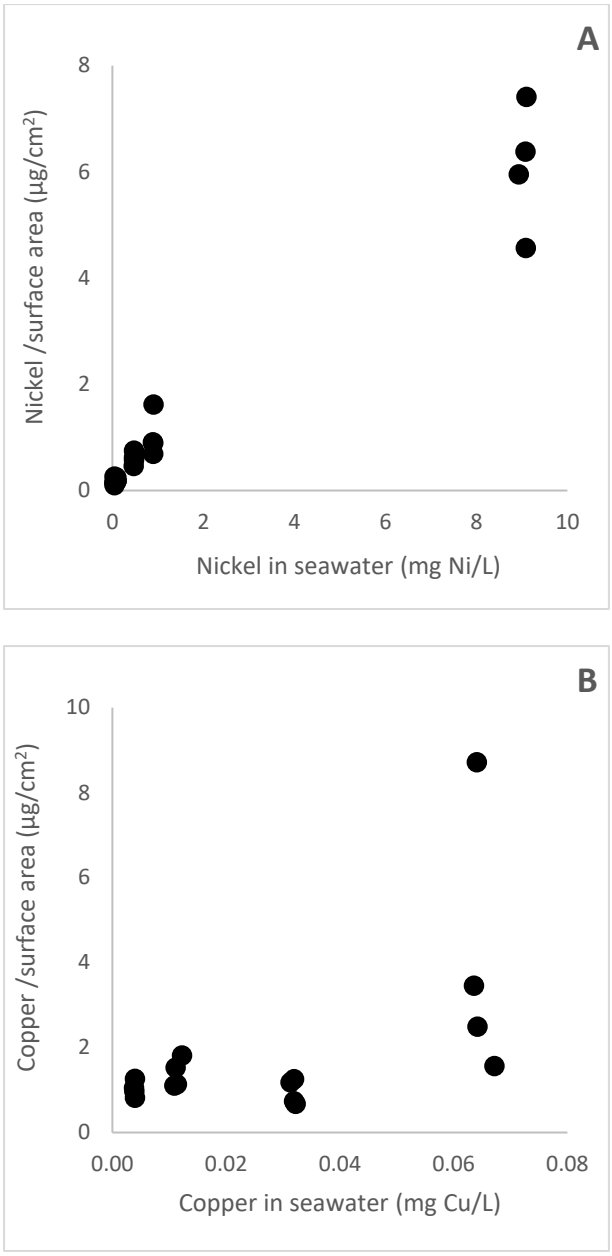



Figure E1. The concentration of nickel (A) and copper (B) per surface area of the coral fragment, versus measured dissolved (0.45-µm) metal concentration in the test chambers. Each point represents one individual fragment from four replicate chambers per treatment. Note the different scales on the x and y axes.

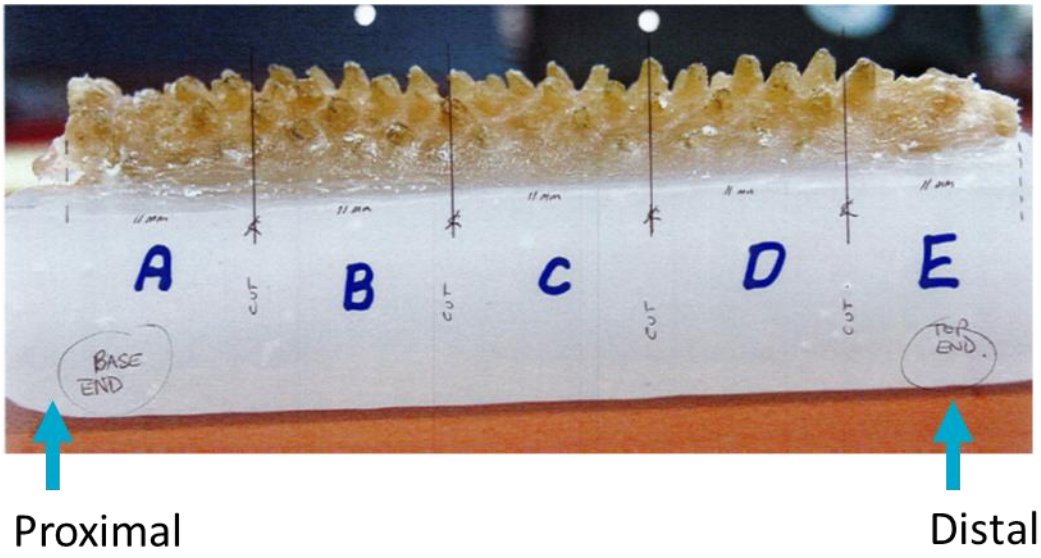



Figure E2. Photograph to demonstrate how the coral fragments were embedded in paraffin wax and cut into sections using a diamond blade cutter prior to analysis with LA-ICPMS

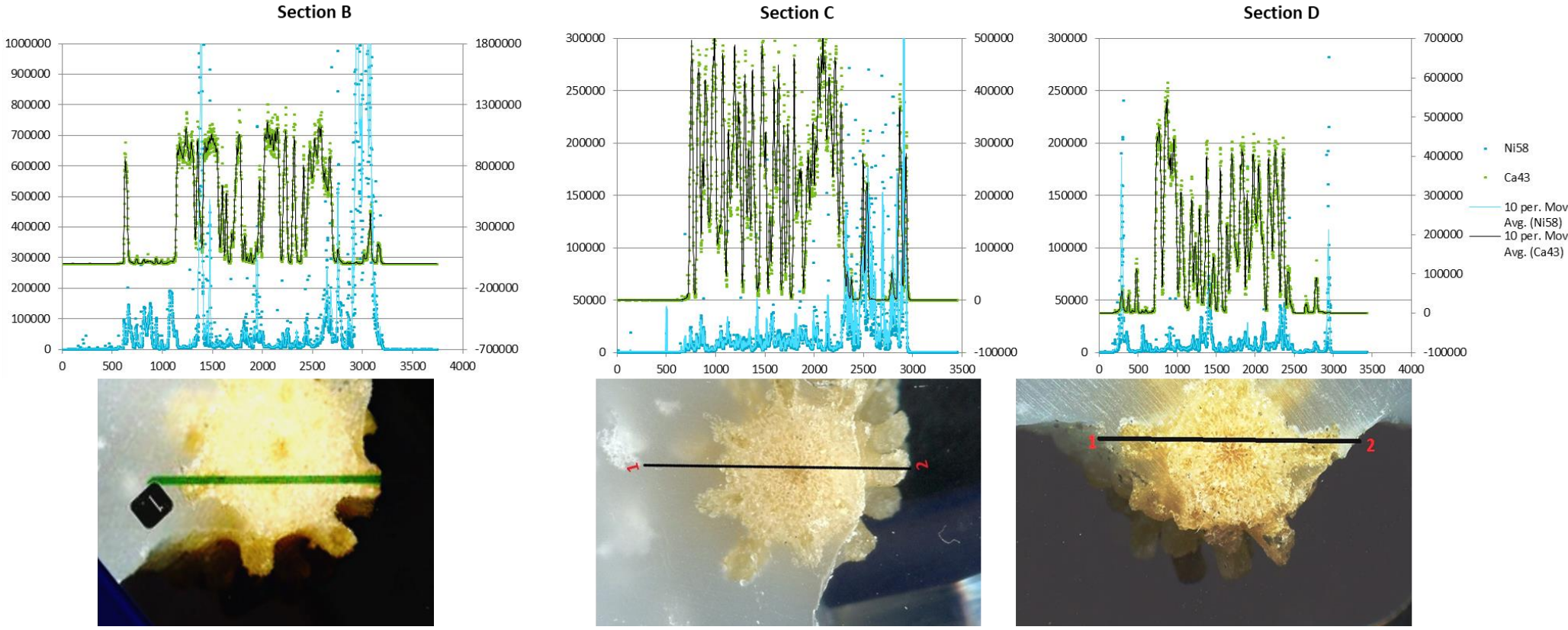



Figure E3. Analysis of a coral fragment exposed to 9050 µg Ni/L (measured, dissolved) by laser-ablation ICPMS. Data show the relative proportion of nickel and calcium detected along the laser line, marked out in the photographs. Measurements on x-axis indicate the location of the laser across the coral section; measurements on the y-axis indicate the number of counts for calcium (left-hand axis) and nickel (right hand axis). Each point is averaged across six data points.

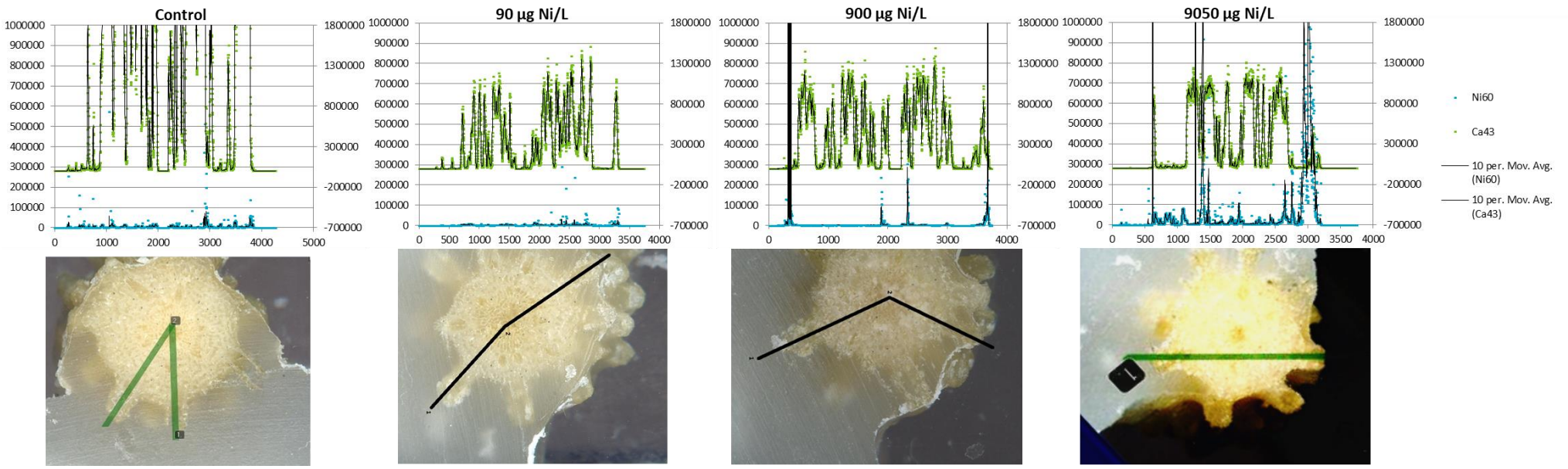



Figure E4. Analysis of a coral fragments exposed to seawater (control), 90, 900 and 9050 µg Ni/L (measured, dissolved) by laser-ablation ICP-MS. Only section B of each fragment was analysed. Data show the relative proportion of nickel and calcium detected along the laser line, marked out in the photographs. Measurements on x-axis indicate the location of the laser across the coral section; measurements on the y-axis indicate the number of counts for calcium (left-hand axis) and nickel (right hand axis). Each point is averaged across six data points.

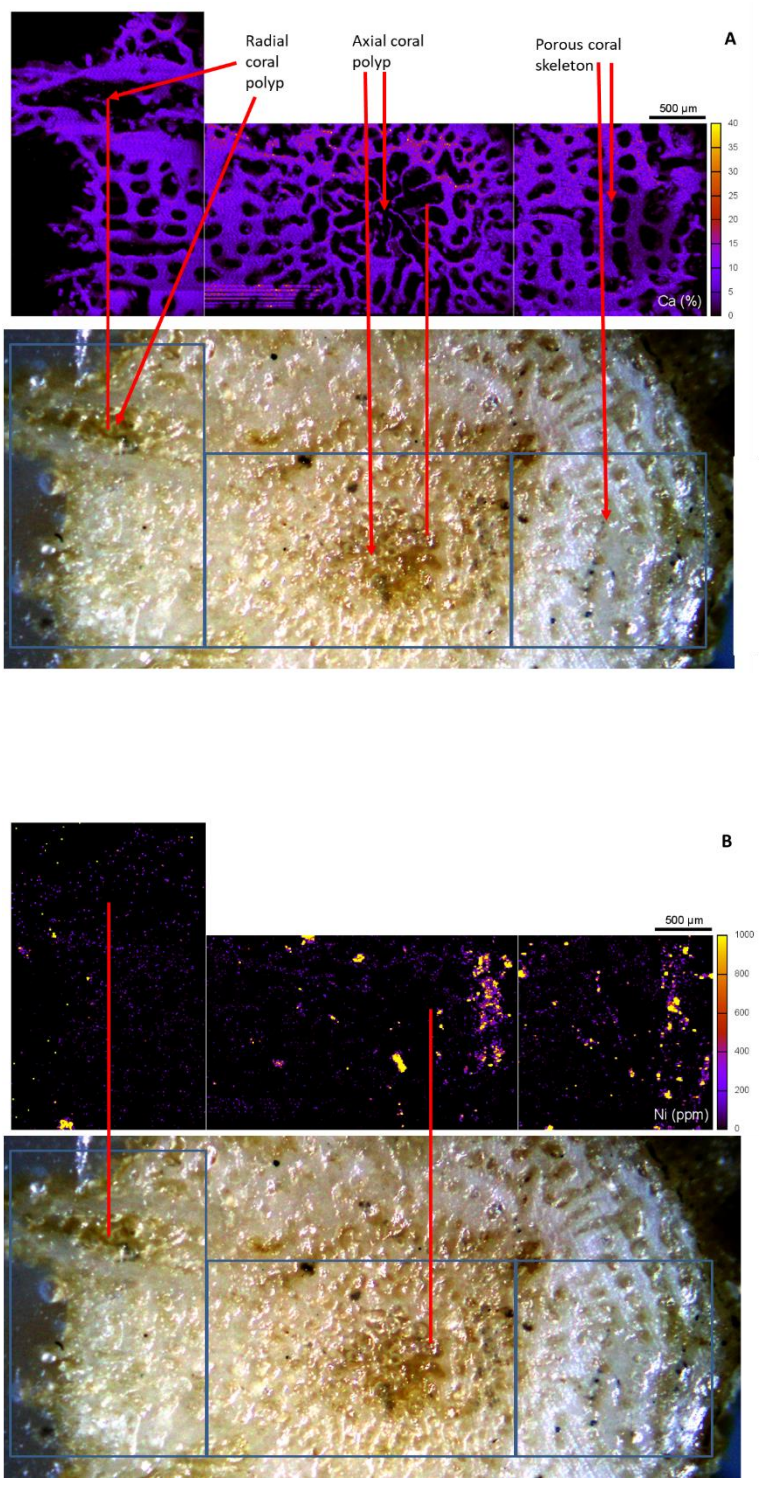
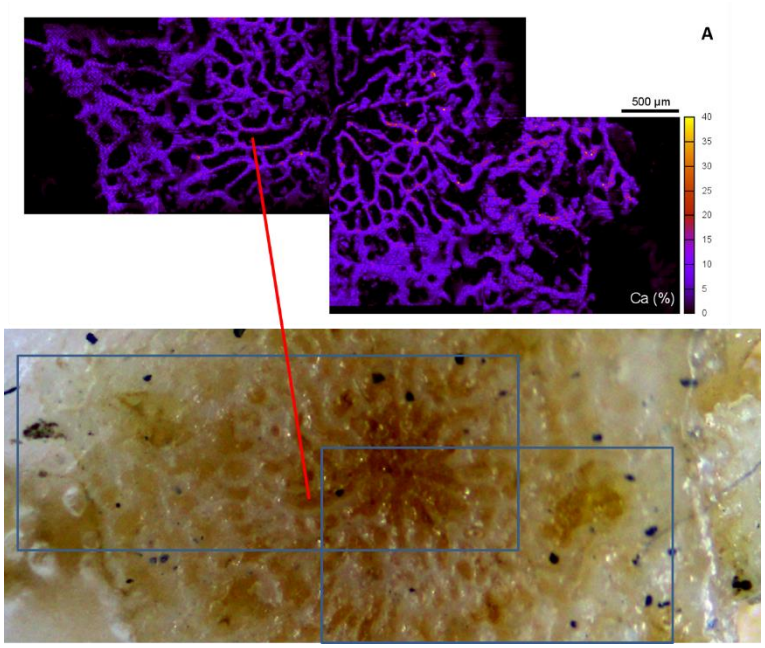



Figure E5. Maps from µ-PIXE analysis showing the detection of calcium (A) and nickel (B) of the Section B (Figure E2) taken from a coral fragment exposed to 9050 µg Ni/L (measured dissolved). The scale bar indicates the relative level of element detected in the sample in the top image. The blue rectangles in the bottom image mark the path of analysis. The upper figure (A) provides an example of the location and image of the external or radial polyps, the central or axial polyp and the porous coral skeleton.



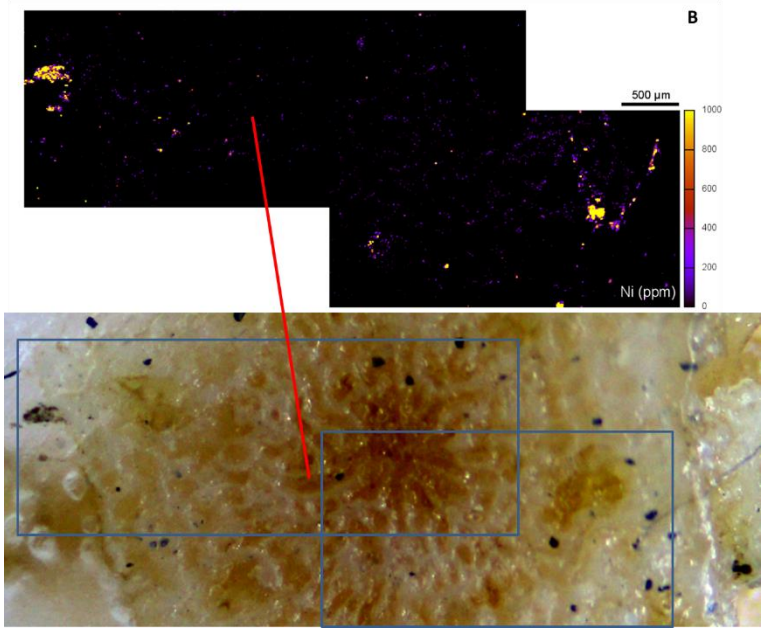




Figure E6. Maps from µ-PIXE analysis showing the detection of calcium (A) and nickel (B) of the Section D (Figure E2) taken from a coral fragment exposed to 9050 µg Ni/L (measured dissolved). The scale bar indicates the relative level of element detected in the sample in the top image. The blue rectangles in the bottom image mark the path of analysis.

**DNA amplification and sequencing using 16S_V5-V6 region primers**

Primers: 784f (5'-3': AGGATTAGATACCCTGGTA), 1061r (5'-3': CRRCACGAGCTGACGAC) for the V5 and V6 region of the 16S rRNA gene (Andersson et al., 2008; Ziegler et al., 2016).PCR methods are described in Chapter 6, Section 6.2.7. All amplifications used the Amplitaq Gold 360 Master Mix (MM, Applied Biosystems), and DNA-free water (brand). For 16S_V5-6 primers the total PCR reaction volume was 50 µL which consisted of 25 µL of MM, 1 µL of each primer, 20 µL of water and 3 µL of template DNA.

PCR conditions:

| | PCR conditions | | |
|---|---|---|---|
| | **Denature** | **Annealing** | **Extension** |
| 16S _V5-6 | 95°C 15 min | 35 cycles: 95°C 20 s, 55°C 90 s, 72°C 60 s | 72°C 7 min |

Total number of reads and operational taxonomic units (OTUs) prior to and after filtering the data in preparation for statistical analysis.

| | Before filtering | | After filtering | |
|---|---|---|---|---|
| **Dataset** | **OTUs** | **Reads** | **OTUs** | **Reads** |
| 16S_V5-6 | 1606 | 1347465 | 207 | 1158830 |

Sequence data was processed through the bioinformatics pipeline and filtered, as per methods in main paper. Statistical analysis followed methods described for 16S in main paper.

*Bacteria – 16S_V5-6*

The general trends observed in the 16S_V4 dataset were similar to the trends in the community structure amplified with the 16S_V5-6 primer set. The bacterial community structure in all nickel treatments were not significantly different to the control. However the highest and lowest nickel treatments were found to be significantly different ($p<0.05$, Figure 13A). The three highest copper treatments were significantly different to the control and the lowest copper treatment (Figure 13B, $p<0.05$).

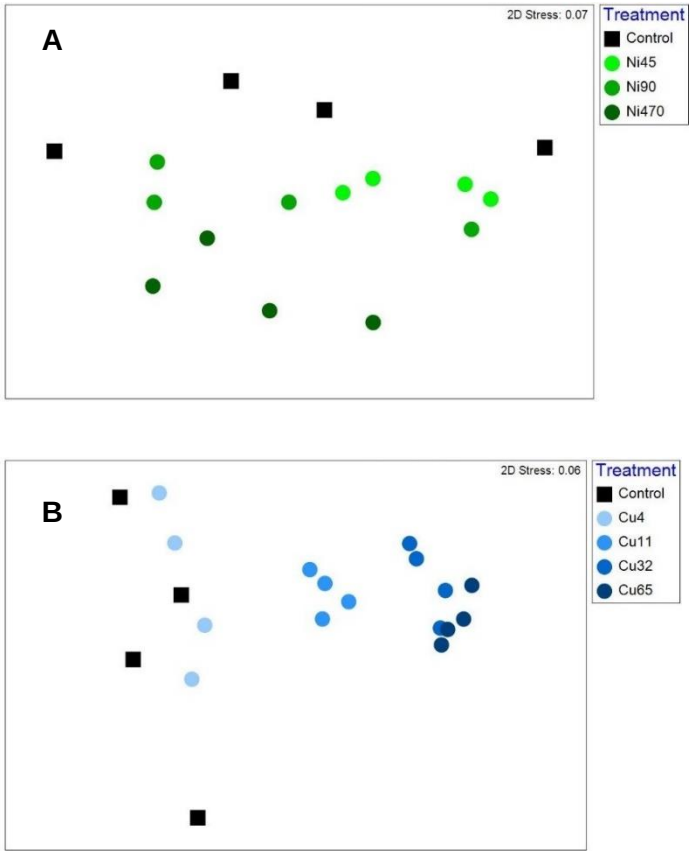



Figure E7. Non-metric multidimensional scaling plots for the 16S_V5-6 dataset for nickel (A) and copper (B). The (dis)similarity between treatments was determined by Bray-Curtis similarity, data were standardised and then square-root transformed. Each point represents one individual replicate from each treatment.

In this dataset, the significant drivers of the differences between the lowest and highest nickel treatment were Rhodobacteraceae (26%) and Hahellaceae (11%). In the copper treatment, the main taxa that were driving the differences between the high copper concentrations and the control and lowest copper were Flavobacteriaceae (21-26%) and Hahellaceae (13-20%) and Rhodobacteraceae (14-17%). Similar to the 16S_V4 dataset, the shade plots in Figure 14 show that there appears to be a slight decrease in the number of Rhodobacteraceae OTUs with increasing nickel concentration. Hahellaceae decreases with increasing copper concentration as Flavobacteriaceae and Rhodobacteraceae OTUs increase (Figure 14). While Planctomycetaceae was detected by the 16S_V5-6 primers, it was not indicated as a key driver of the differences between the bacterial communities in the copper or nickel treatments, unlike the 16S_V4 primers.

# Appendix F. Coral Microbiomes altered by Exposure to Sediment and Nickel

Table F1. Correlation between turbidity (Formanzin nephelometric units, FNU) measured by the turbidity meters connected to the experimental tanks and TSS concentration (mg/L) determined by gravimetric analysis for clean-sediment, Ni-sediment and field-sediment (C). Data shown are from one representative analysis. Measurements taken across multiple time-points did not vary significantly from the examples shown. Two replicate samples were taken from each experimental tank

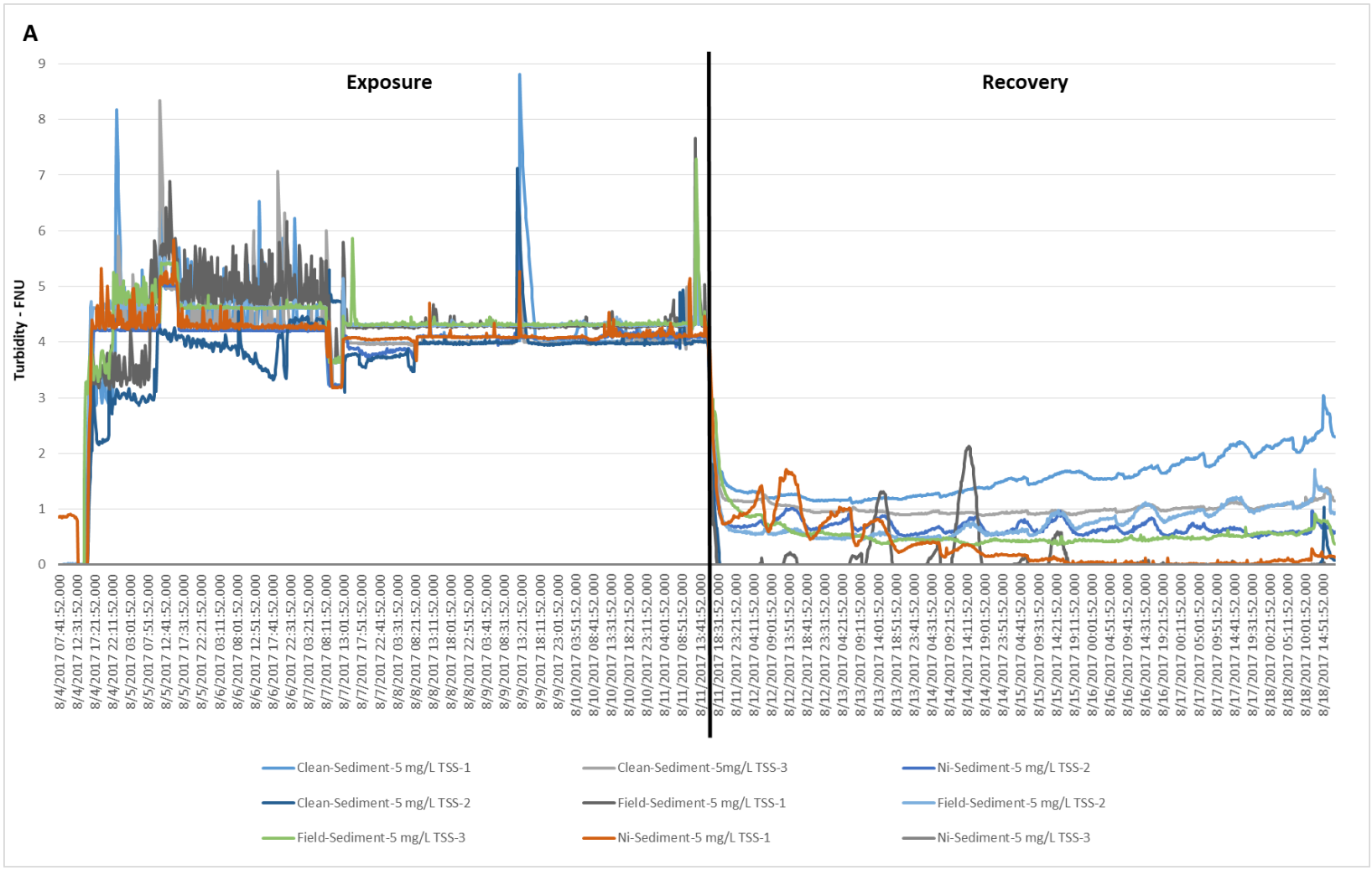
| Treatment | Replicate tank | FNU | TSS (mg/L) | Ratio of TSS/FNU |
|---|---|---|---|---|
| Clean-sediment, 5 mg TSS/L | 1 | 4.0 | 3.6 | 0.9 |
| Clean-sediment, 5 mg TSS/L | 1 | 4.1 | 3.7 | 0.9 |
| Clean-sediment, 5 mg TSS/L | 2 | 3.8 | 4.6 | 1.2 |
| Clean-sediment, 5 mg TSS/L | 2 | 3.6 | 4.5 | 1.2 |
| Clean-sediment, 5 mg TSS/L | 3 | 4.1 | 4.1 | 1.0 |
| Clean-sediment, 5 mg TSS/L | 3 | 4.0 | 4.0 | 1.0 |
| Clean-sediment, 30 mg TSS/L | 1 | 24 | 30 | 1.2 |
| Clean-sediment, 30 mg TSS/L | 1 | 24 | 32 | 1.3 |
| Clean-sediment, 30 mg TSS/L | 2 | 23 | 28 | 1.2 |
| Clean-sediment, 30 mg TSS/L | 2 | 24 | 27 | 1.1 |
| Clean-sediment, 30 mg TSS/L | 3 | 24 | 28 | 1.2 |
| Clean-sediment, 30 mg TSS/L | 3 | 24 | 28 | 1.2 |
| Ni-sediment, 5 mg TSS/L | 1 | 4.1 | 4.0 | 1.0 |
| Ni-sediment, 5 mg TSS/L | 1 | 4.1 | 4.0 | 1.0 |
| Ni-sediment, 5 mg TSS/L | 2 | 3.9 | 4.4 | 1.1 |
| Ni-sediment, 5 mg TSS/L | 2 | 3.9 | 4.2 | 1.1 |
| Ni-sediment, 5 mg TSS/L | 3 | 4.1 | 4.0 | 1.0 |
| Ni-sediment, 5 mg TSS/L | 3 | 4.1 | 4.0 | 1.0 |
| Ni-sediment, 30 mg TSS/L | 1 | 24 | 28 | 1.2 |
| Ni-sediment, 30 mg TSS/L | 1 | 24 | 28 | 1.2 |
| Ni-sediment, 30 mg TSS/L | 2 | 24 | 30 | 1.2 |
| Ni-sediment, 30 mg TSS/L | 2 | 24 | 30 | 1.2 |
| Ni-sediment, 30 mg TSS/L | 3 | 24 | 29 | 1.2 |
| Ni-sediment, 30 mg TSS/L | 3 | 24 | 31 | 1.3 |
| Field-sediment, 5 mg TSS/L | 1 | 4.3 | 5.7 | 1.3 |
| Field-sediment, 5 mg TSS/L | 1 | 4.3 | 5.9 | 1.4 |
| Field-sediment, 5 mg TSS/L | 2 | 4.3 | 4.2 | 1.0 |
| Field-sediment, 5 mg TSS/L | 2 | 4.3 | 4.1 | 1.0 |
| Field-sediment, 5 mg TSS/L | 3 | 4.4 | 5.0 | 1.2 |
| Field-sediment, 30 mg TSS/L | 3 | 4.4 | 4.9 | 1.1 |
| Field-sediment, 30 mg TSS/L | 1 | 26 | 29 | 1.1 |
| Field-sediment, 30 mg TSS/L | 1 | 26 | 30 | 1.2 |
| Field-sediment, 30 mg TSS/L | 2 | 26 | 28 | 1.1 |
| Field-sediment, 30 mg TSS/L | 2 | 26 | 29 | 1.1 |
| Field-sediment, 30 mg TSS/L | 3 | 26 | 31 | 1.2 |
| Field-sediment, 30 mg TSS/L | 3 | 26 | 31 | 1.2 |

Table F2. Total number of reads and operational taxonomic units (OTUs) prior to and after filtering the data in preparation for statistical analysis.

| | Before filtering | | After filtering | |
|---|---|---|---|---|
| **Dataset** | **OTUs** | **Reads** | **OTUs** | **Reads** |
| 18S rDNA | 374 | 5301074 | 59 | 4438549 |
| 16S rDNA | 4691 | 2416954 | 589 | 1311743 |
| ITS2 rDNA | 15 | 4394421 | 12 | 4117038 |

Table F3. Dissolved organic carbon concentrations in treatment tanks measured during the exposure period (t=0-7 d), measured in one replicate tank per treatment, rounded to 2 significant figures

| **Sample name** | **Day 0 (4/08/17)** | **Day 6 (10/08/17)** | **Average** | **Std. deviation** |
|---|---|---|---|---|
| Control | 1.10 | 1.10 | 1.10 | 0.04 |
| 200 µg Ni/L | 1.30 | 1.10 | 1.20 | 0.20 |
| 400 µg Ni/L | 1.20 | 1.20 | 1.20 | 0.01 |
| Clean-sediment, 5 mg TSS/L | 1.50 | 1.10 | 1.30 | 0.30 |
| Clean-sediment, 30 mg TSS/L | 1.408 | 1.10 | 1.20 | 0.20 |
| Ni-sediment, 5 mg TSS/L | 1.20 | 1.30 | 1.20 | 0.05 |
| Ni-sediment, 30 mg TSS/L | 1.30 | 1.10 | 1.20 | 0.20 |
| Field-sediment, 5 mg TSS/L | 1.40 | 1.30 | 1.40 | 0.05 |
| Field-sediment, 30 mg TSS/L | 1.20 | 1.50 | 1.30 | 0.20 |


A
Exposure
Recovery
Turbidity - FNU
0
1
2
3
4
5
6
7
8
9
8/4/2017 07:41:52.000
8/4/2017 12:31:52.000
8/4/2017 17:21:52.000
8/4/2017 22:11:52.000
8/5/2017 03:01:52.000
8/5/2017 07:51:52.000
8/5/2017 12:41:52.000
8/5/2017 17:31:52.000
8/5/2017 22:21:52.000
8/6/2017 03:11:52.000
8/6/2017 08:01:52.000
8/6/2017 12:51:52.000
8/6/2017 17:41:52.000
8/6/2017 22:31:52.000
8/7/2017 03:21:52.000
8/7/2017 08:11:52.000
8/7/2017 13:01:52.000
8/7/2017 17:51:52.000
8/7/2017 22:41:52.000
8/8/2017 03:31:52.000
8/8/2017 08:21:52.000
8/8/2017 13:11:52.000
8/8/2017 18:01:52.000
8/8/2017 22:51:52.000
8/9/2017 03:41:52.000
8/9/2017 08:31:52.000
8/9/2017 13:21:52.000
8/9/2017 18:11:52.000
8/9/2017 23:01:52.000
8/10/2017 03:51:52.000
8/10/2017 08:41:52.000
8/10/2017 13:31:52.000
8/10/2017 18:21:52.000
8/10/2017 23:11:52.000
8/11/2017 04:01:52.000
8/11/2017 08:51:52.000
8/11/2017 13:41:52.000
8/11/2017 18:31:52.000
8/11/2017 23:21:52.000
8/12/2017 04:11:52.000
8/12/2017 09:01:52.000
8/12/2017 13:51:52.000
8/12/2017 18:41:52.000
8/12/2017 23:31:52.000
8/13/2017 04:21:52.000
8/13/2017 09:11:52.000
8/13/2017 14:01:52.000
8/13/2017 18:51:52.000
8/13/2017 23:41:52.000
8/14/2017 04:31:52.000
8/14/2017 09:21:52.000
8/14/2017 14:11:52.000
8/14/2017 19:01:52.000
8/14/2017 23:51:52.000
8/15/2017 04:41:52.000
8/15/2017 09:31:52.000
8/15/2017 14:21:52.000
8/15/2017 19:11:52.000
8/16/2017 00:01:52.000
8/16/2017 04:51:52.000
8/16/2017 09:41:52.000
8/16/2017 14:31:52.000
8/16/2017 19:21:52.000
8/17/2017 00:11:52.000
8/17/2017 05:01:52.000
8/17/2017 09:51:52.000
8/17/2017 14:41:52.000
8/17/2017 19:31:52.000
8/18/2017 00:21:52.000
8/18/2017 05:11:52.000
8/18/2017 10:01:52.000
8/18/2017 14:51:52.000
Clean-Sediment-5 mg/L TSS-1
Clean-Sediment-5mg/L TSS-3
Ni-Sediment-5 mg/L TSS-2
Clean-Sediment-5 mg/L TSS-2
Field-Sediment-5 mg/L TSS-1
Field-Sediment-5 mg/L TSS-2
Field-Sediment-5 mg/L TSS-3
Ni-Sediment-5 mg/L TSS-1
Ni-Sediment-5 mg/L TSS-3

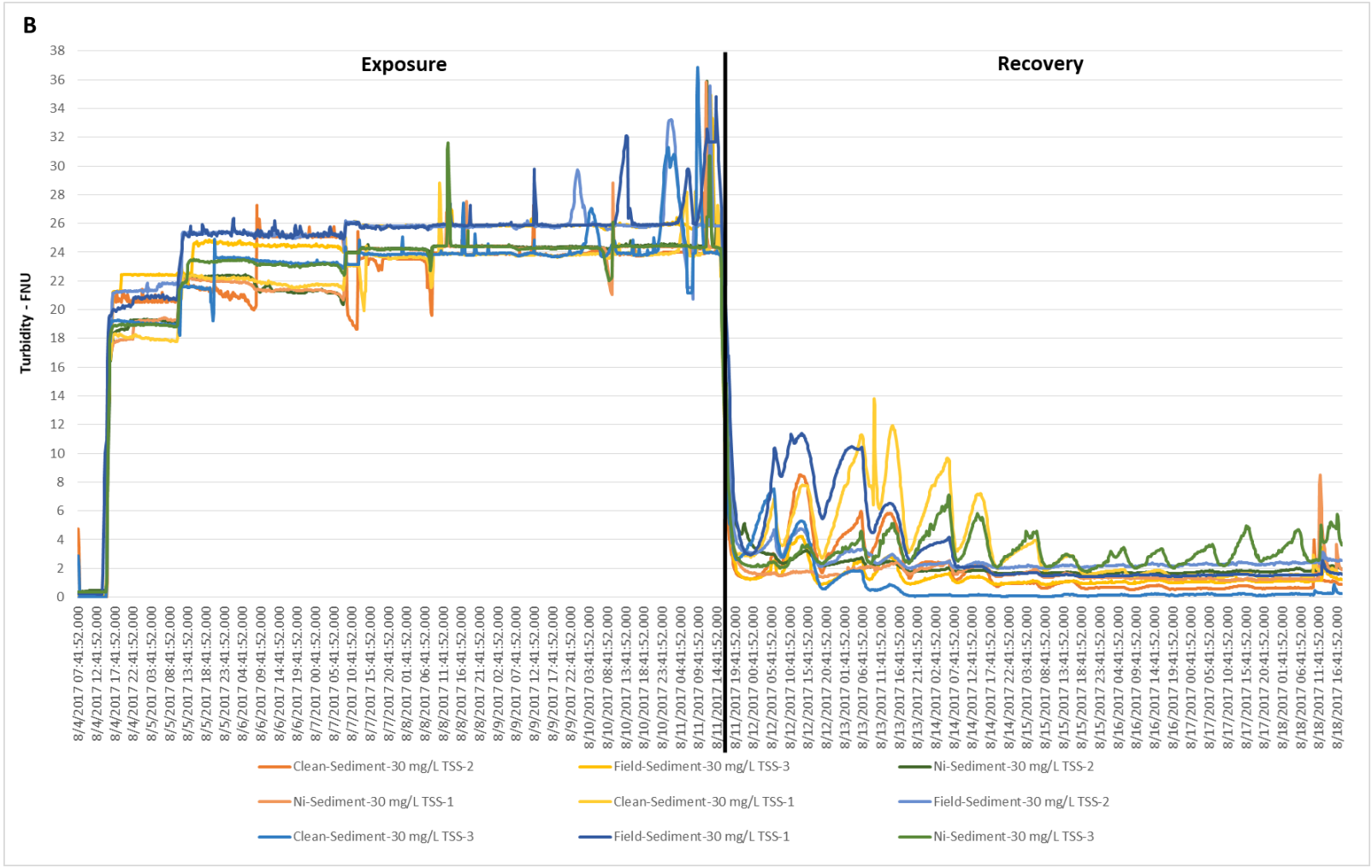



Figure F1. Real time measurements of turbidity (FNU) in sediment treatment tanks during the exposure and recovery period at ~5 mg TSS/L (A) and ~30 mg TSS/L (B)

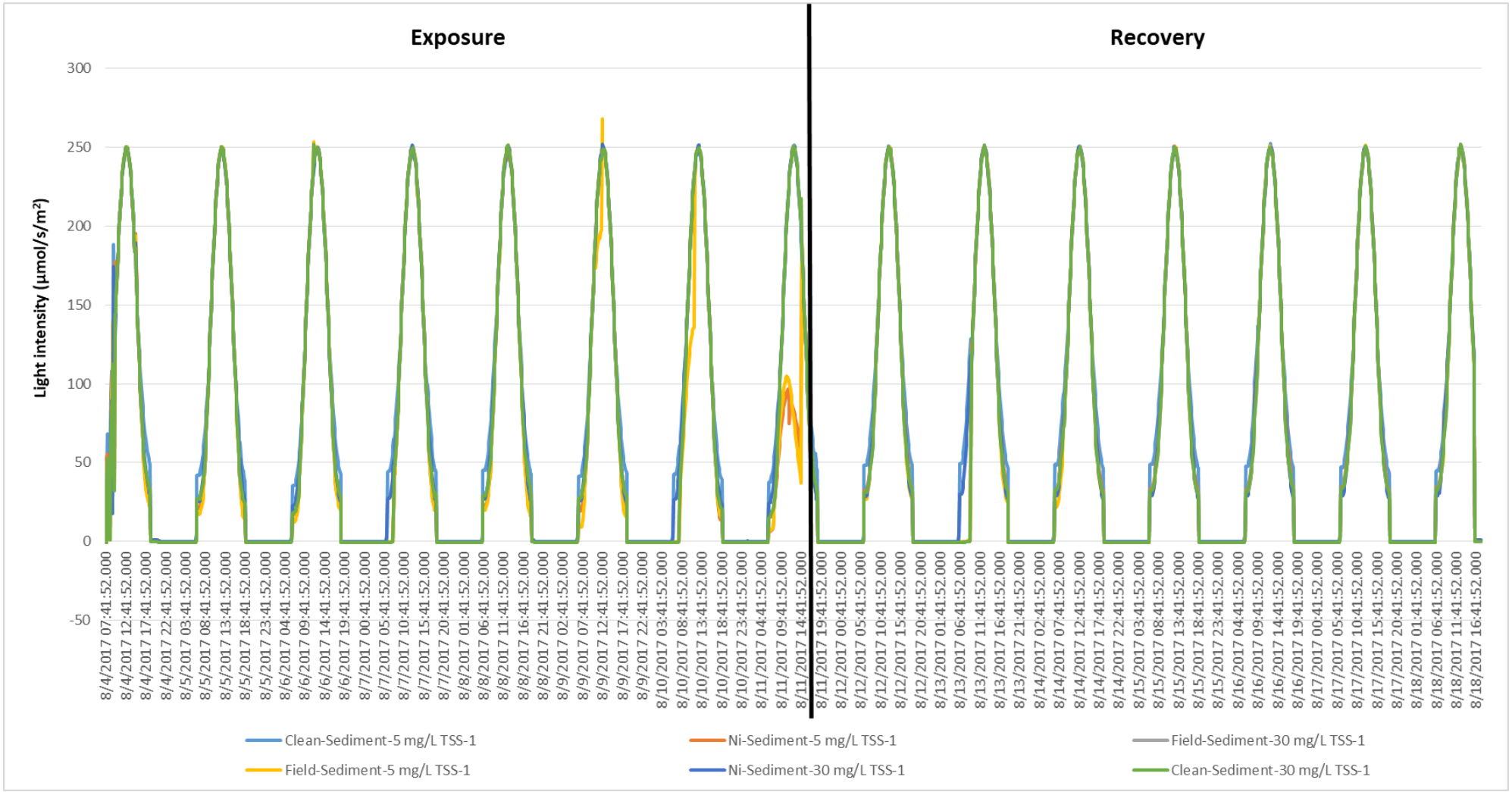



Figure F2. Light measurements taken in tanks during experimental period

## Analysis of coral microbiomes sampled from the field, aquaria and laboratory

Table F4. PERMANOVA Pair-wise tests for 16s rDNA and 18S rDNA datasets to compare the microbiome of corals sampled: (A) from the field, (B) from the aquaria (following 6-week acclimation) and (C) following 24 h in experimental tanks

| **16S rDNA** | | | |
|---|---|---|---|
| **Groups** | **t** | **P(perm)** | **Unique perms** |
| A, B | 2.618 | 0.0026 | 462 |
| A, C | 3.1772 | 0.0004 | 3932 |
| B, C | 1.0045 | 0.4238 | 6881 |
| **18S rDNA** | | | |
| **Groups** | **t** | **P(perm)** | **Unique perms** |
| A, B | 2.9275 | 0.0028 | 245 |
| A, C | 2.9114 | 0.0002 | 6965 |
| B, C | 0.61743 | 0.8045 | 6941 |

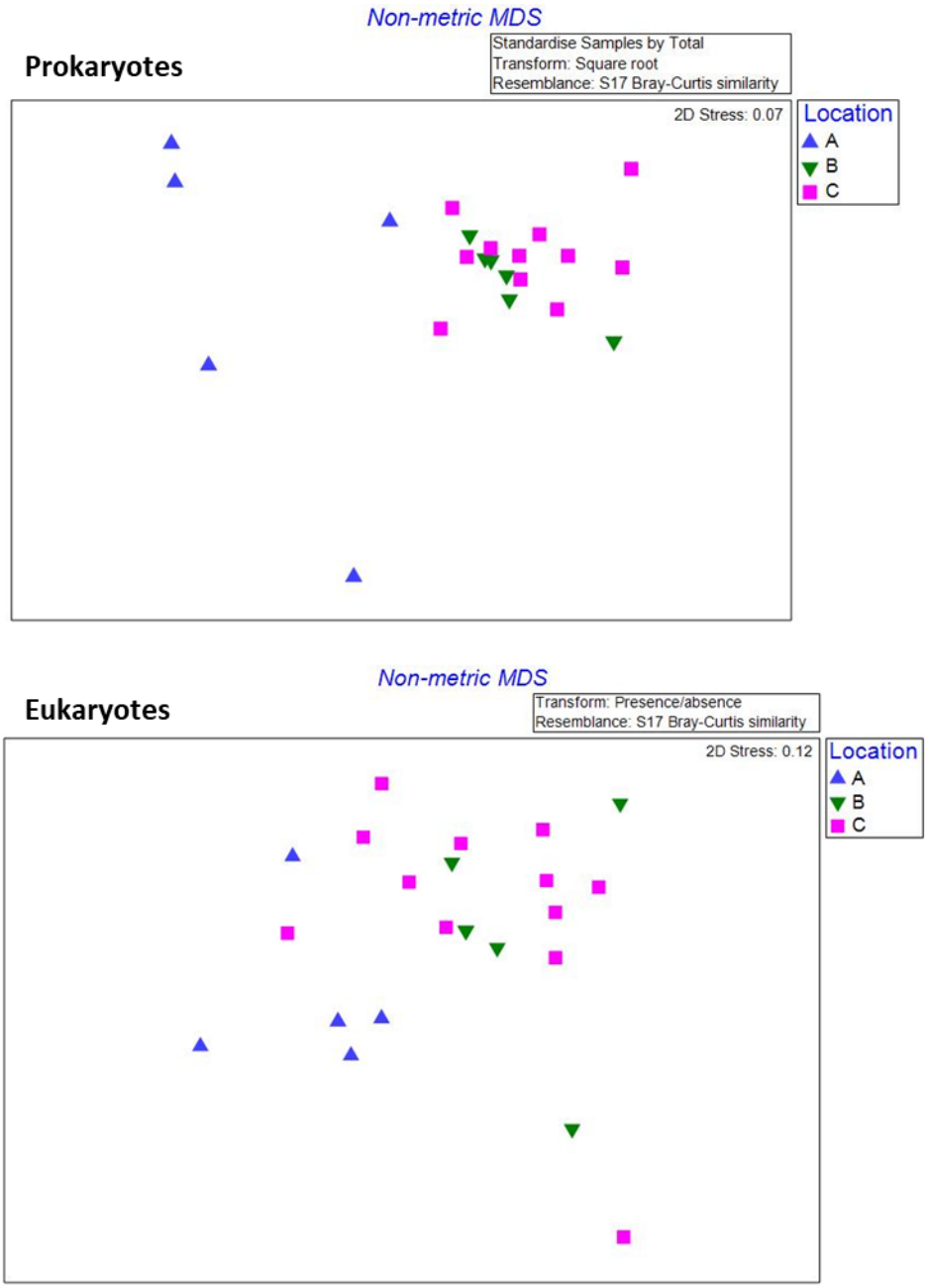



Figure F3. Non metric dimensional scaling plots showing the relative (dis) similarties in the prokaryote and eukaryote community composition of the coral microbiome sampled: (A) from the field, (B) from the aquaria (following 6-week acclimation), and (C) following 24 h in experimental tanks.

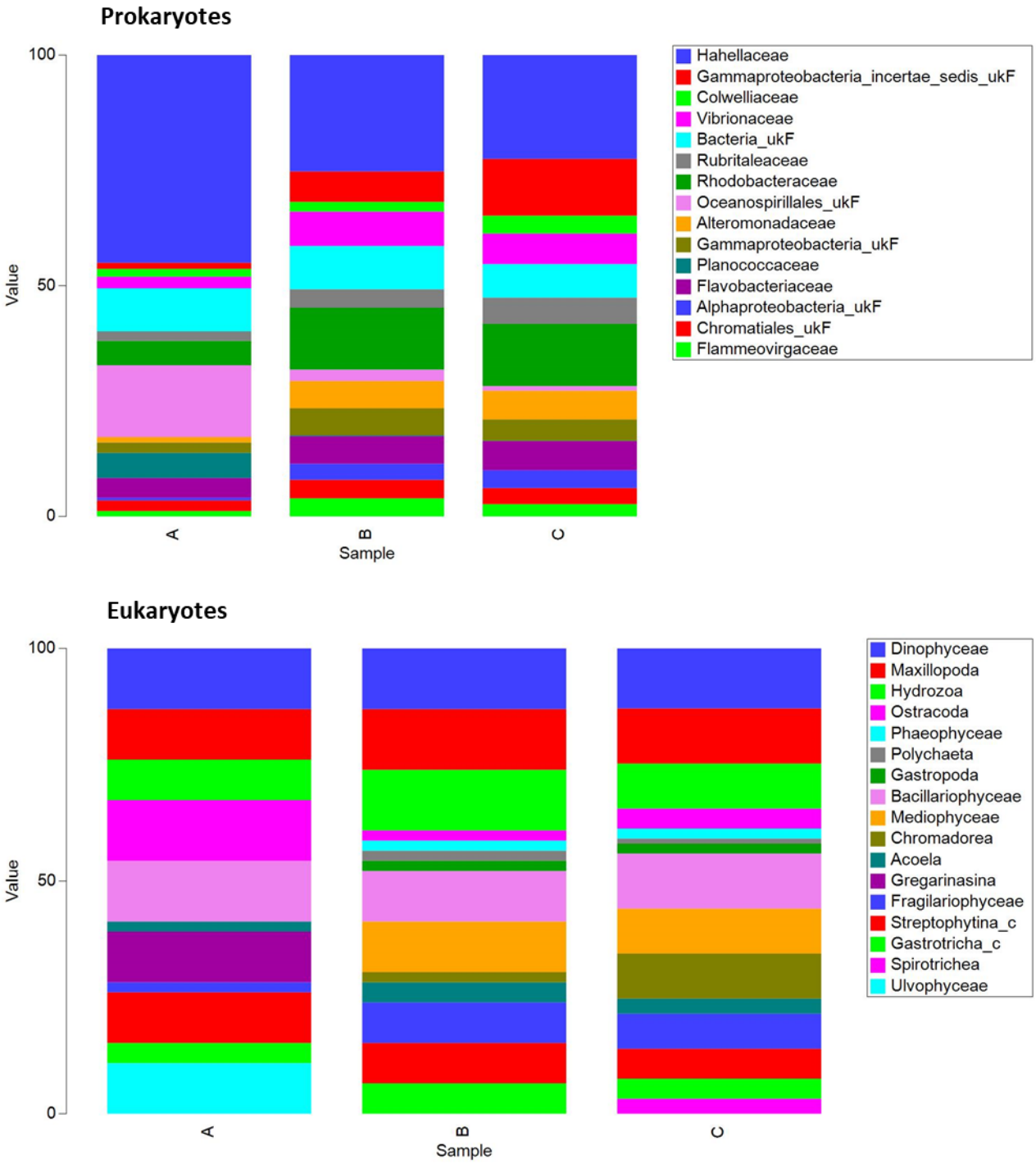



Figure F4. Bar plots showing the community composition of the prokaryote and eukaryote community of the coral microbiome sampled: (A) from the field, (B) from the aquaria (following 6-week acclimation) and (C) following 24 h in experimental tanks

Table F5. Main test PERMANOVA results for the 18S rDNA, ITS2 (*Symbiodinium*) and 16S rDNA datasets for the dissolved nickel treatments. df = degrees of freedom, SS = sum of squares, MS = mean squares.

| **18S rDNA** | | | | | | |
|---|---|---|---|---|---|---|
| Source | df | SS | MS | Pseudo-F | P(perm) | Unique perms |
| Treatment (Ni µg/L) | 2 | 694.79 | 347.39 | 2.4061 | 0.1331 | 15 |
| Time (days) | 2 | 1154.7 | 577.37 | 1.8026 | 0.1092 | 9957 |
| tank(treatment) | 3 | 433.15 | 144.38 | 0.37172 | 0.9559 | 9924 |
| treatmentxtime | 4 | 690.31 | 172.58 | 0.53881 | 0.9119 | 9950 |
| timextank(treatment) | 6 | 1921.8 | 320.3 | 0.82463 | 0.6591 | 9917 |
| Residual | 18 | 6991.4 | 388.41 | | | |
| Total | 35 | 11886 | | | | |
| **ITS2 (*Symbiodinium*)** | | | | | | |
| Source | df | SS | MS | Pseudo-F | P(perm) | Unique perms |
| Treatment (Ni µg/L) | 2 | 460.59 | 230.29 | 3.0113 | 0.1945 | 15 |
| Time (days) | 2 | 553.79 | 276.89 | 3.4481 | **0.0457** | 9963 |
| tank(treatment) | 3 | 229.43 | 76.476 | 0.65581 | 0.7084 | 9949 |
| treatmentxtime | 4 | 512.58 | 128.15 | 1.5958 | 0.2214 | 9951 |
| timextank(treatment) | 6 | 481.82 | 80.304 | 0.68864 | 0.7812 | 9916 |
| Residual | 18 | 2099 | 116.61 | | | |
| Total | 35 | 4337.2 | | | | |
| **16S rDNA** | | | | | | |
| Source | df | SS | MS | Pseudo-F | P(perm) | Unique perms |
| Treatment (Ni µg/L) | 2 | 1563.3 | 781.65 | 2.2315 | 0.065 | 15 |
| Time (days) | 2 | 2344.1 | 1172 | 3.0056 | **0.0008** | 9930 |
| tank(treatment) | 3 | 1050.8 | 350.28 | 0.82751 | 0.696 | 9895 |
| treatmentxtime | 4 | 2433.8 | 608.46 | 1.5603 | 0.0782 | 9917 |
| timextank(treatment) | 6 | 2339.7 | 389.95 | 0.92124 | 0.6096 | 9870 |
| Residual | 18 | 7619.2 | 423.29 | | | |
| Total | 35 | 17351 | | | | |

Table F6. Pairwise PERMANOVA results for 16S rDNA dataset comparing different time points, t = 4, 7 and 14 d for the dissolved nickel treatments

| **Groups** | **t** | **P(perm)** | **Unique perms** |
|---|---|---|---|
| t=4 d, t=7 d | 1.243 | 0.2156 | 9946 |
| t=4 d, t=14 d | 1.8397 | **0.0066** | 9930 |
| t=7 d, t=14 d | 2.2593 | **0.0001** | 9921 |

Table F7. Main test PERMANOVA results for the 18S rDNA, ITS2 (*Symbiodinium*) and 16S rDNA datasets for the Field sediment treatments. df = degrees of freedom, SS = sum of squares, MS = mean squares.

| **18S rDNA** | | | | | | |
|---|---|---|---|---|---|---|
| **Source** | **df** | **SS** | **MS** | **Pseudo-F** | **P(perm)** | **Unique perms** |
| Treatment (TSS, mg/L) | 1 | 418.35 | 418.35 | 1.1321 | 0.4109 | 10 |
| Time (d) | 2 | 2068.7 | 1034.4 | 3.7687 | **0.0024** | 9938 |
| tank(treatment) | 4 | 1478.2 | 369.54 | 0.87951 | 0.5866 | 9923 |
| treatmentxtime | 2 | 387.48 | 193.74 | 0.7059 | 0.7014 | 9926 |
| timextank(treatment) | 8 | 2195.7 | 274.46 | 0.65321 | 0.9156 | 9890 |
| Residual | 18 | 7563.1 | 420.17 | | | |
| Total | 35 | 14111 | | | | |
| **ITS2 (*Symbiodinium*)** | | | | | | |
| Source | df | SS | MS | Pseudo-F | P(perm) | Unique perms |
| Treatment (TSS, mg/L) | 1 | 149.35 | 149.35 | 3.3471 | 0.2034 | 10 |
| Time (d) | 2 | 82.249 | 41.124 | 0.59844 | 0.6441 | 9956 |
| tank(treatment) | 4 | 178.48 | 44.621 | 0.37916 | 0.9076 | 9946 |
| treatmentxtime | 2 | 193.13 | 96.563 | 1.4052 | 0.2817 | 9962 |
| timextank(treatment) | 8 | 549.75 | 68.719 | 0.58392 | 0.8457 | 9929 |
| Residual | 18 | 2118.3 | 117.68 | | | |
| Total | 35 | 3271.3 | | | | |
| **16S rDNA** | | | | | | |
| Source | df | SS | MS | Pseudo-F | P(perm) | Unique perms |
| Treatment (TSS, mg/L) | 1 | 757.33 | 757.33 | 2.2158 | 0.1047 | 60 |
| Time (d | 2 | 2985.4 | 1492.7 | 6.4627 | **0.0001** | 9937 |
| tank(treatment) | 4 | 1366.4 | 341.59 | 0.91671 | 0.5572 | 9920 |
| treatmentxtime | 2 | 300.43 | 150.22 | 0.65037 | 0.7915 | 9941 |
| timextank(treatment) | 8 | 1839.7 | 229.96 | 0.61714 | 0.9559 | 9895 |
| Residual | 13 | 4844.1 | 372.62 | | | |
| Total | 30 | 12123 | | | | |

Table F8. Pairwise PERMANOVA results for 18S and 16S rDNA dataset comparing different time points, t, of 4, 7 and 14 d for Field-sediment treatments

| **18S rDNA** | | | |
|---|---|---|---|
| Groups | t | P(perm) | Unique perms |
| t=4 d, t=7 d | 1.8527 | **0.029** | 9939 |
| t=4 d, t=14 d | 2.1364 | **0.018** | 9934 |
| t=7 d, t=14 d | 1.8075 | **0.0298** | 9934 |
| 16S rDNA | | | |
| Groups | t | P(perm) | Unique perms |
| t=4 d, t=7 d | 2.4596 | **0.0019** | 9915 |
| t=4 d, t=14 d | 2.7845 | **0.0003** | 9940 |
| t=7 d, t=14 d | 2.4272 | **0.0004** | 9937 |

Table F9. PERMANOVA results for ITS2 dataset, df = degrees of freedom, SS = sum of squares, MS = mean squares, for the clean- and Ni-sediment

| Source | df | SS | MS | Pseudo-F | P(perm) | Unique perms |
|---|---|---|---|---|---|---|
| Matrix (Sediment type) | 1 | 165.53 | 165.53 | 2.2592 | 0.1467 | 8930 |
| Treatment (TSS, mg/L) | 1 | 29.569 | 29.569 | 0.40356 | 0.5864 | 8891 |
| Time (days) | 2 | 203.5 | 101.75 | 1.0215 | 0.3893 | 9962 |
| matrix x treatment | 1 | 223.59 | 223.59 | 3.0516 | 0.1144 | 8941 |
| matrix x time | 2 | 73.102 | 36.551 | 0.36696 | 0.7886 | 9945 |
| treatment x time | 2 | 149.48 | 74.739 | 0.75035 | 0.5161 | 9949 |
| tank (matrix x treatment) | 8 | 586.15 | 73.269 | 1.1173 | 0.3529 | 9937 |
| matrix x treatment x time | 2 | 218.97 | 109.49 | 1.0992 | 0.3575 | 9939 |
| time x tank (matrix x treatment) | 16 | 1593.7 | 99.605 | 1.5189 | 0.1112 | 9924 |
| Residual | 36 | 2360.7 | 65.576 | | | |
| Total | 71 | 5604.3 | | | | |

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